

**GENE ENCODING A PROTEIN INVOLVED IN THE SIGNAL TRANSDUCTION
CASCADE LEADING TO SYSTEMIC ACQUIRED RESISTANCE IN PLANTS**

This application is a continuation of U.S. Application No. 09/577,799, filed May 24, 2000,
5 which is a continuation of U.S. Application No. 08/880,179, filed June 20, 1997, issued as U.S.
Patent No. 6,091,004, which claims the benefit of the following U.S. Provisional Applications: U.S.
Provisional Application No. 60/020,272, filed June 21, 1996, U.S. Provisional Application No.
60/024,883, filed August 30, 1996, U.S. Provisional Application No. 60/033,177, filed December
13, 1996, U.S. Provisional Application No. 60/034,379, filed December 27, 1996, U.S. Provisional
10 Application No. 60/034,730, filed January 10, 1997, and U.S. Provisional Application No.
60/035,022, filed January 10, 1997. The disclosures of each of these U.S. Provisional Applications
are hereby expressly incorporated by reference in their entireties into the instant disclosure.

FIELD OF THE INVENTION

15 The present invention relates to broad-spectrum disease resistance in plants, including the
phenomenon of systemic acquired resistance (SAR). More particularly, the present invention relates
to the identification, isolation and characterization of a gene involved in the signal transduction
cascade leading to systemic acquired resistance.

BACKGROUND OF THE INVENTION

20 Plants are constantly challenged by a wide variety of pathogenic organisms including
viruses, bacteria, fungi, and nematodes. Crop plants are particularly vulnerable because they are
25 usually grown as genetically-uniform monocultures; when disease strikes, losses can be severe.
However, most plants have their own innate mechanisms of defense against pathogenic organisms.
Natural variation for resistance to plant pathogens has been identified by plant breeders and
pathologists and bred into many crop plants. These natural disease resistance genes often provide
high levels of resistance to or immunity against pathogens.

Systemic acquired resistance (SAR) is one component of the complex system plants use to defend themselves from pathogens (Hunt and Ryals 1996; Ryals et al., 1996). SAR is a particularly important aspect of plant-pathogen responses because it is a pathogen-inducible, systemic resistance against a broad spectrum of infectious agents, including viruses, bacteria, and fungi. When the SAR signal transduction pathway is blocked, plants become more susceptible to pathogens that normally cause disease, and they also become susceptible to some infectious agents that would not normally cause disease (Gaffney et al., 1993; Delaney et al., 1994, 1995; 1993; Bi et al., 1995; Mauch-Mani and Slusarenko 1996; Delaney 1997). These observations indicate that the SAR signal transduction pathway is critical for maintaining plant health.

Conceptually, the SAR response can be divided into two phases. In the initiation phase, a pathogen infection is recognized, and a signal is released that travels through the phloem to distant tissues. This systemic signal is perceived by target cells, which react by expression of both SAR genes and disease resistance. The maintenance phase of SAR refers to the period of time, from weeks up to the entire life of the plant, during which the plant is in a quasi steady state, and disease resistance is maintained (Ryals et al., 1996).

Salicylic acid (SA) accumulation appears to be required for SAR signal transduction. Plants that cannot accumulate SA due to treatment with specific inhibitors, epigenetic repression of phenylalanine ammonia-lyase, or transgenic expression of salicylate hydroxylase, which specifically degrades SA, also cannot induce either SAR gene expression or disease resistance (Gaffney et al., 1993; Delaney et al., 1994; Maher et al., 1994; Mauch-Mani and Slusarenko 1996; Pallas et al., 1996). *See also*, U.S. Patent No. 5,804,693, incorporated herein by reference in its entirety). Although it has been suggested that SA might serve as the systemic signal, this is currently controversial and, to date, all that is known for certain is that if SA cannot accumulate, then SAR signal transduction is blocked (Pallas et al., 1996; Shulaev et al., 1995; Vernooij et al., 1994).

Recently, Arabidopsis has emerged as a model system to study SAR (Uknes et al., 1992; Uknes et al., 1993; Cameron et al., 1994; Mauch-Mani and Slusarenko 1994; Dempsey and Klessig 1995). It has been demonstrated that SAR can be activated in Arabidopsis by both pathogens and chemicals, such as SA, 2,6-dichloroisonicotinic acid (INA) and benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) (Uknes et al., 1992; Vernooij et al., 1995; Lawton et al.,

1996). Following treatment with either INA or BTH or pathogen infection, at least three pathogenesis-related (PR) protein genes, namely, *PR-1*, *PR-2*, and *PR-5* are coordinately induced concomitant with the onset of resistance (Uknes et al., 1992, 1993). In tobacco, the best characterized species, treatment with a pathogen or an immunization compound induces the expression of at least nine sets of genes (Ward et al., 1991).

A number of Arabidopsis mutants have been isolated that have modified SAR signal transduction. The first of these mutants are the so-called *lsd* (lesions simulating disease) mutants and *acd2* (accelerated cell death) (Dietrich et al., 1994; Greenberg et al., 1994). These mutants all have some degree of spontaneous necrotic lesion formation on their leaves, elevated levels of SA, mRNA accumulation for the SAR genes, and significantly enhanced disease resistance. At least seven different *lsd* mutants have been isolated and characterized (Dietrich et al., 1994; Weymann et al., 1995). Another interesting class of mutants are *cim* (constitutive immunity) mutants (Lawton et al., 1993; Steiner et al., 1996). *See also*, U.S. Patent No. 5,792,904, hereby incorporated by reference into the instant disclosure in its entirety. Like *lsd* mutants and *acd2*, *cim* mutants have elevated SA and SAR gene expression and resistance, but in contrast to *lsd* or *acd2*, do not display detectable lesions on their leaves. *cpr1* (constitutive expresser of PR genes) may be a type of *cim* mutant; however, because the presence of microscopic lesions on the leaves of *cpr1* has not been ruled out, *cpr1* might be a type of *lsd* mutant (Bowling et al., 1994).

Mutants have also been isolated that are blocked in SAR signaling. *ndr1* (non-race-specific disease resistance) is a mutant that allows growth of both *Pseudomonas syringae* containing various avirulence genes and also normally avirulent isolates of *Peronospora parasitica* (Century et al., 1995). Apparently this mutant is blocked early in SAR signaling.

Despite much research and the use of sophisticated and intensive crop-protection measures, including genetic transformation of plants, losses due to disease remain in the billions of dollars annually. Disease resistance genes have previously been cloned but transgenic plants transformed with these genes are typically resistant only to a subset of strains of a particular pathogen species. Despite efforts to clone genes involved in SAR, a gene controlling broad spectrum disease resistance has not until now been isolated and characterized.

SUMMARY OF THE INVENTION

The present invention concerns the identification, isolation, and characterization of the *NIM1* gene, which encodes a protein involved in the signal transduction cascade responsive to biological and chemical inducers that leads to systemic acquired resistance in plants.

Hence, the present invention is directed to an isolated DNA molecule (*NIM1* gene) that encodes a NIM1 protein involved in the signal transduction cascade leading to systemic acquired resistance in plants.

In one preferred embodiment, the DNA molecule that encodes the NIM1 protein hybridizes under the following conditions to clone BAC-04, ATCC Deposit No. 97543: hybridization in 1%BSA; 520mM NaPO₄, pH7.2; 7% lauryl sulfate, sodium salt; 1mM EDTA; 250 mM sodium chloride at 55°C for 18-24h, and wash in 6XSSC for 15 min. (X3) 3XSSC for 15 min. (X1) at 55°C. In an especially preferred embodiment, the *NIM1* gene is comprised within clone BAC-04, ATCC Deposit No. 97543.

In another embodiment, the DNA molecule that encodes the NIM1 protein hybridizes under the following conditions to cosmid D7, ATCC Deposit No. 97736: hybridization in 1%BSA; 520mM NaPO₄, pH7.2; 7% lauryl sulfate, sodium salt; 1mM EDTA; 250 mM sodium chloride at 55°C for 18-24h, and wash in 6XSSC for 15 min. (X3) 3XSSC for 15 min. (X1) at 55°C. In an especially preferred embodiment, the *NIM1* gene is comprised within cosmid D7, ATCC Deposit No. 97736.

The *NIM1* gene of the invention may be isolated from a dicotyledonous plant such as *Arabidopsis*, tobacco, cucumber, or tomato. Alternately, the *NIM1* gene may be isolated from a monocotyledonous plant such as maize, wheat, or barley.

In yet another embodiment, the encoded NIM1 protein comprises the amino acid sequence set forth in SEQ ID NO:3. In still another embodiment, the *NIM1* gene coding sequence hybridizes under the following conditions to the coding sequence set forth in SEQ ID NO:2: hybridization in 1%BSA; 520mM NaPO₄, pH7.2; 7% lauryl sulfate, sodium salt; 1mM EDTA; 250 mM sodium chloride at 55°C for 18-24h, and wash in 6XSSC for 15 min. (X3) 3XSSC for 15 min. (X1) at 55°C. In an especially preferred embodiment, the *NIM1* gene coding sequence comprises the coding sequence set forth in SEQ ID NO:2.

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The present invention also encompasses a chimeric gene comprising a promoter active in plants operatively linked to a *NIM1* gene coding sequence, a recombinant vector comprising such a chimeric gene, wherein the vector is capable of being stably transformed into a host, as well as a host stably transformed with such a vector. Preferably, the host is a plant such as one of the following agronomically important crops: rice, wheat, barley, rye, corn, potato, carrot, sweet potato, sugar beet, bean, pea, chicory, lettuce, cabbage, cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tobacco, tomato, sorghum and sugarcane.

In an especially preferred embodiment, the NIM1 protein is expressed in a transformed plant at higher levels than in a wild type plant.

The present invention is also directed to a method of conferring a CIM phenotype to a plant by transforming the plant with a recombinant vector comprising a chimeric gene that itself comprises a promoter active in plants operatively linked to a *NIM1* gene coding sequence, wherein the encoded NIM1 protein is expressed in the transformed plant at higher levels than in a wild type plant.

Further, the present invention is directed to a method of activating systemic acquired resistance in a plant by transforming the plant with a recombinant vector comprising a chimeric gene that itself comprises a promoter active in plants operatively linked to a *NIM1* gene coding sequence, wherein the encoded NIM1 protein is expressed in the transformed plant at higher levels than in a wild type plant.

In addition, the present invention is directed to a method of conferring broad spectrum disease resistance to a plant by transforming the plant with a recombinant vector comprising a chimeric gene that itself comprises a promoter active in plants operatively linked to a *NIM1* gene coding sequence, wherein the encoded NIM1 protein is expressed in the transformed plant at higher levels than in a wild type plant.

In yet another aspect, the present invention is directed to a method of screening for a *NIM1* gene involved in the signal transduction cascade leading to systemic acquired resistance in a plant, comprising probing a genomic or cDNA library from said plant with a *NIM1* coding sequence that

hybridizes under the following set of conditions to the coding sequence set forth in SEQ ID NO:2: hybridization in 1%BSA; 520mM NaPO₄, pH7.2; 7% lauryl sulfate, sodium salt; 1mM EDTA; 250 mM sodium chloride at 55°C for 18-24h, and wash in 6XSSC for 15 min. (X3) 3XSSC for 15 min. (X1) at 55°C.

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BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows the effect of chemical inducers on the induction of SAR gene expression in wild-type and *nim1* plants. Chemical induction of SAR genes is diminished in *nim1* plants. Water, SA, INA, or BTH is applied to wild type (WT) and *nim1* plants. After 3 days, RNA is prepared from these plants and examined for expression of PR-1, PR-2, and PR-5.

FIGURE 2 depicts PR-1 gene expression in pathogen-infected Ws-O and *nim1* plants. Pathogen induction of PR-1 is diminished in *nim1* plants. Wild type (WT) and *nim1* plants were spray-inoculated with the Emwa race of *P. parasitica*. Samples were collected at days 0, 1, 2, 4, and 6 and RNA is analyzed by blot hybridization with an *A. thaliana* PR-1 cDNA probe to measure PR-1 mRNA accumulation.

FIGURE 3 shows the accumulation of PR-1 mRNA in *nim1* mutants and wild-type plants after pathogen infection or chemical treatment. Plants containing *nim1* alleles *nim1*-1, -2, -3, -4, -5, and -6 and Ws-O (Ws) were treated with water (C), SA, INA, or BTH 3 days before RNA isolation. The Emwa sample consists of RNA isolated from plants 14 days post-inoculation with the Emwa isolate of *P. parasitica*. Blots were hybridized using an *Arabidopsis* PR-1 cDNA as a probe (Uknes *et al.*, 1992).

FIGURE 4 shows the levels of SA accumulation in Ws-O and *nim1* plants infected with *P. syringae*. *nim1* plants accumulate SNA following pathogen exposure. Leaves of wild type and *nim1* plants are infiltrated with *Pst* DC3000(*avrRpt2*) or carrier medium (10 mM MgCl₂) alone. After 2 days, samples were collected from untreated, MgCl₂-treated, and DC3000(*avrRpt2*)-treated plants. Bacteria-treated samples were separated into primary (infiltrated) and secondary (noninfiltrated) leaves. Free SA and total SA following hydrolysis with β -glucosidase were quantified by HPLC. Error bars indicate SD of three replicate samples.

FIGURES 5A-D present a global map at increasing levels of resolution of the chromosomal region centered on *NIM1* with recombinants indicated, including, BACs, YACs and Cosmids in *NIM1* region.

- (A) Map position of *NIM1* on chromosome 1. The total number of gametes scored is 2276.
- (B) Yeast artificial chromosome (striped), bacterial artificial chromosome (BAC), and P1 clones used to clone *NIM1*.
- (C) Cosmid clones that cover the *NIM1* locus. The three cosmids that complement *nim1-1* are shown as thicker lines.
- (D) The four putative gene regions on the smallest fragment of complementing genomic DNA. The four open reading frames that comprise the *NIM1* gene are indicated by the open bars. The arrows indicate the direction of transcription. Numbering is relative to the first base of *Arabidopsis* genomic DNA present in cosmid D7.

FIGURE 6 shows the nucleic acid sequence of the *NIM1* gene and the amino acid sequence of the *NIM1* gene product, including changes in the various alleles. This nucleic acid sequence, which is on the opposite strand as the 9.9 kb sequence presented in SEQ ID NO:1, is also presented in SEQ ID NO:2, and the amino acid sequence of the *NIM1* gene product is also presented in SEQ ID NO:3.

FIGURE 7 shows the accumulation of *NIM1* induced by INA, BTH, SA and pathogen treatment in wild type plants and mutant alleles of *nim1*. The RNA gel blots in Figure 3 were probed for expression of RNA by using a probe derived from 2081 to 3266 in the sequence shown in Figure 6.

FIGURE 8 is an amino acid sequence comparison of Expressed Sequence Tag regions of the NIM1 protein and cDNA protein products of 4 rice gene sequences.

BRIEF DESCRIPTION OF THE SEQUENCES IN THE SEQUENCE LISTING

SEQ ID NO:1 - 9919-bp genomic sequence of *NIM1* gene region 2 in Figure 5D .

SEQ ID NO:2 - 5655-bp genomic sequence in Figure 6 (opposite strand from SEQ ID NO:1).

5 SEQ ID NO:3 - AA sequence of wild-type NIM1 protein encoded by cds of SEQ ID NO:2.

SEQ ID NO:4 - Rice-1 AA sequence 33-155 from Figure 8.

SEQ ID NO:5 - Rice-1 AA sequence 215-328 from Figure 8.

SEQ ID NO:6 - Rice-2 AA sequence 33-155 from Figure 8.

SEQ ID NO:7 - Rice-2 AA sequence 208-288 from Figure 8.

10 SEQ ID NO:8 - Rice-3 AA sequence 33-155 from Figure 8.

SEQ ID NO:9 - Rice-3 AA sequence 208-288 from Figure 8.

SEQ ID NO:10 - Rice-4 AA sequence 33-155 from Figure 8.

SEQ ID NO:11 - Rice-4 AA sequence 215-271 from Figure 8.

SEQ ID NO:12 - Oligonucleotide.

15 SEQ ID NO:13 - Oligonucleotide.

SEQ ID NO:14 - Oligonucleotide.

SEQ ID NO:15 - Oligonucleotide.

SEQ ID NO:16 - Oligonucleotide.

SEQ ID NO:17 - Oligonucleotide.

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DETAILED DESCRIPTION OF THE INVENTION

Definitions

25	<i>acd</i> :	accelerated cell death mutant plant
	AFLP:	Amplified Fragment Length Polymorphism
	avrRpt2:	avrulence gene Rpt2, isolated from <i>Pseudomonas syringae</i>
	BAC:	Bacterial Artificial Chromosome
	BTH:	benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester
30	CIM:	<u>C</u> onstitutive <u>I</u> mmunity phenotype (SAR is constitutively activated)

	<i>cim</i> :	constitutive <u>i</u> mmunity mutant plant
	cM:	centimorgans
	<i>cpr1</i> :	constitutive expresser of <u>P</u> R genes mutant plant
	Col-O:	<i>Arabidopsis</i> ecotype Columbia
5	ECs:	Enzyme combinations
	Emwa:	<i>Peronospora parasitica</i> isolate compatible in the Ws-O ecotype of <i>Arabidopsis</i>
	EMS:	ethyl methane sulfonate
	INA:	2,6-dichloroisonicotinic acid
	Ler:	<i>Arabidopsis</i> ecotype <i>Landsberg erecta</i>
10	<i>lsd</i> :	lesions <u>s</u> imulating <u>d</u> isease mutant plant
	<i>nahG</i> :	salicylate hydroxylase <i>Pseudomonas putida</i> that converts salicylic acid to catechol
	NahG:	<i>Arabidopsis</i> line transformed with <i>nahG</i> gene
	<i>ndr</i> :	<u>n</u> on-race-specific <u>d</u> isease <u>r</u> esistance mutant plant
	<i>nim</i> :	<u>n</u> on-inducible <u>i</u> mmunity mutant plant
15	<i>NIM1</i> :	the wild type gene, involved in the SAR signal transduction cascade
	NIM1:	Protein encoded by the wild type <i>NIM1</i> gene
	<i>nim1</i> :	mutant allele of <i>NIM1</i> , conferring disease susceptibility to the plant; also refers to mutant <i>Arabidopsis thaliana</i> plants having the <i>nim1</i> mutant allele of <i>NIM1</i>
	Noco:	<i>Peronospora parasitica</i> isolate compatible in the Col-O ecotype of <i>Arabidopsis</i>
20	ORF:	open reading frame
	PCs:	Primer combinations
	PR:	Pathogenesis Related
	SA:	salicylic acid
	SAR:	Systemic Acquired Resistance
25	SSLP:	Simple Sequence Length Polymorphism
	UDS:	Universal Disease Susceptible phenotype
	Wela:	<i>Peronospora parasitica</i> isolate compatible in the Weiningen ecotype of <i>Arabidopsis</i>
	Ws-O:	<i>Arabidopsis</i> ecotype Issilewskija
	WT:	wild type
30	YAC:	Yeast Artificial Chromosome

The *nim* Mutant Phenotype

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The present invention relates to mutant plants, as well as genes isolated therefrom, which are defective in their normal response to pathogen infection in that they do not express genes associated with SAR. These mutants are referred to as *nim* mutants (for non-inducible immunity) and are "universal disease susceptible" (UDS) by virtue of their being susceptible to many strains and pathotypes of pathogens of the host plant and also to pathogens that do not normally infect the host plant, but that normally infect other hosts. Such mutants can be selected by treating seeds or other biological material with mutagenic agents and then selecting progeny plants for the UDS phenotype by treating progeny plants with known chemical inducers (e.g. INA) of SAR and then infecting the plants with a known pathogen. Non-inducible mutants develop severe disease symptoms under these circumstances, whereas wild type plants are induced by the chemical compound to systemic acquired resistance. *nim* mutants can be equally selected from mutant populations generated by chemical and irradiation mutagenesis, as well as from populations generated by T-DNA insertion and transposon-induced mutagenesis. Techniques of generating mutant plant lines are well known in the art.

nim mutants provide useful indicators of the evaluation of disease pressure in field pathogenesis tests where the natural resistance phenotype of so-called wild type (i.e. non-mutant) plants may vary and therefore not provide a reliable standard of susceptibility. Furthermore, *nim* plants have additional utility for the testing of candidate disease resistance transgenes. Using a *nim* stock line as a recipient for transgenes, the contribution of the transgene to disease resistance is directly assessable over a base level of susceptibility. Furthermore, the *nim* plants are useful as a tool in the understanding of plant-pathogen interactions. *nim* host plants do not mount a systemic response to pathogen attack, and the unabated development of the pathogen is an ideal system in which to study its biological interaction with the host.

As *nim* host plants may also be susceptible to pathogens outside of the host-range they normally fall, these plants also have significant utility in the molecular, genetic, and biological study of host-pathogen interactions. Furthermore, the UDS phenotype of *nim* plants also renders them of utility for fungicide screening. *nim* mutants selected in a particular host have considerable utility for the screening of fungicides using that host and pathogens of the host. The advantage lies

in the UDS phenotype of the mutant, which circumvents the problems encountered by hosts being differentially susceptible to different pathogens and pathotypes, or even resistant to some pathogens or pathotypes.

nim mutants have further utility for the screening of fungicides against a range of pathogens and pathotypes using a heterologous host, i.e. a host that may not normally be within the host species range of a particular pathogen. Thus, the susceptibility of *nim* mutants of *Arabidopsis* to pathogens of other species (e.g. crop plant species) facilitates efficacious fungicide screening procedures for compounds against important pathogens of crop plants.

The *Arabidopsis thaliana nim1* Mutant

An *Arabidopsis thaliana* mutant called *nim1* (noninducible immunity) that supports *P. parasitica* (i.e., causal agent of downy mildew disease) growth following INA treatment is described in Delaney et al., 1995. Although *nim1* can accumulate SA following pathogen infection, neither SAR gene expression nor disease resistance can be induced, suggesting that the mutation blocks the pathway downstream of SA. *nim1* is also impaired in its ability to respond to INA or BTH, suggesting that the block exists downstream of the action of these chemicals (Delaney et al., 1995; Lawton et al., 1996). This first *Arabidopsis nim1* mutant (herein designated *nim1-1*) was isolated from 80,000 plants of a T-DNA tagged *Arabidopsis* ecotype Issilewskija (Ws-0) population by spraying two week old plants with 0.33 mM INA followed by inoculation with *P. parasitica* (Delaney et al., 1995). Plants that supported fungal growth after INA treatment were selected as putative mutants. Five additional mutants (herein designated *nim1-2*, *nim1-3*, *nim1-4*, *nim1-5*, and *nim1-6*) were isolated from 280,000 M₂ plants from an ethyl methanesulfonate (EMS)-mutagenized Ws-0 population.

To determine whether the mutants were dominant or recessive, Ws-0 plants were used as pollen donors to cross to each of these mutants. The F₁ plants were then scored for their ability to support fungal growth following INA treatment. As shown in Table 3 of the Examples, all *nim1-1*, *nim1-2*, *nim1-3*, *nim1-4*, and *nim1-6* F₁ plants were phenotypically wild type, indicating a recessive mutation in each line. *nim1-5* showed the *nim* phenotype in all 35 F₁ plants, indicating that this particular mutant is dominant. For verification, the reciprocal cross was carried out using *nim1-5* as

the pollen donor to fertilize Ws-0 plants. In this case, all 18 F₁ plants were phenotypically *nim*, confirming the dominance of the *nim1-5* mutation.

To determine whether the *nim1-2* through *nim1-6* mutations were allelic to the previously characterized *nim1-1* mutation, pollen from *nim1-1* was used to fertilize *nim1-2* through *nim1-6*.

5 Because *nim1-1* carried resistance to kanamycin, F₁ progeny were identified by antibiotic resistance. In all cases, the kanamycin-resistant F₁ plants were *nim*, indicating they were all allelic to the *nim1-1* mutant. Because the *nim1-5* mutant is dominant and apparently homozygous for the mutation, it was necessary to analyze *nim1-1* complementation in the F₂ generation. If *nim1-1* and *nim1-5* were allelic, then the expectation would be that all F₂ plants have a *nim* phenotype. If not, 10 then 13 of 16 F₂ plants would have been expected to have a *nim* phenotype. Of 94 plants, 88 clearly supported fungal growth following INA treatment. Six plants showed an associated phenotype of black specks on the leaves reminiscent of a lesion mimic phenotype and supported little fungal growth following INA treatment. Because *nim1-5* carries a point mutation in the *NIM1* gene (*infra*), it is considered to be a *nim1* allele.

15 To determine the relative strength of the different *nim1* alleles, each mutant was analyzed for the growth of *P. parasitica* under normal growth conditions and following pretreatment with either SA, INA, or BTH. As shown in Table 1, during normal growth, *nim1-1*, *nim1-2*, *nim1-3*, *nim1-4*, and *nim1-6* all supported approximately the same rate of fungal growth, which was somewhat faster than the Ws-0 control. The exception was the *nim1-5* plants, in which fungal growth was delayed by several days relative to both the other *nim1* mutants and the Ws-0 control, 20 but eventually all of the *nim1-5* plants succumbed to the fungus. Following SA treatment, the mutants could be grouped into three classes: *nim1-4* and *nim1-6* showed a relatively rapid fungal growth; *nim1-1*, *nim1-2*, *nim1-3* plants exhibited a somewhat slower rate of fungal growth; and fungal growth in *nim1-5* plants was even slower than in the untreated Ws-0 controls. Following 25 either INA or BTH treatment, the mutants also seemed to fall into three classes where *nim1-4* was the most severely compromised in its ability to restrict fungal growth following chemical treatment; *nim1-1*, *nim1-2*, *nim1-3*, and *nim1-6* were all moderately compromised; and *nim1-5* was only slightly compromised. In these experiments, Ws-0 did not support fungal growth following INA or BTH treatment. Thus, with respect to inhibition of fungal growth following chemical treatment, the 30 mutants fall into three classes with *nim1-4* being the most severely compromised, *nim1-1*, *nim1-2*,

nim1-3 and *nim1-6* showing an intermediate inhibition of fungus and *nim1-5* with only slightly impaired fungal resistance.

The accumulation of *PR-1* mRNA was also used as a criterion to characterize the different *nim1* alleles. RNA was extracted from plants 3 days after either water or chemical treatment, or 14 days after inoculation with a compatible fungus (*P. parasitica* isolate Emwa). The RNA gel blot in Figure 3 shows that *PR-1* mRNA accumulated to high levels following treatment of wild-type plants with SA, INA, or BTH or infection by *P. parasitica*. In the *nim1-1*, *nim1-2*, and *nim1-3* plants, *PR-1* mRNA accumulation was dramatically reduced relative to the wild type following chemical treatment. *PR-1* mRNA was also reduced following *P. parasitica* infection, but there was still some accumulation in these mutants. In the *nim1-4* and *nim1-6* plants, *PR-1* mRNA accumulation was more dramatically reduced than in the other alleles following chemical treatment (evident in longer exposures) and significantly less *PR-1* mRNA accumulated following *P. parasitica* infection, supporting the idea that these could be particularly strong *nim1* alleles. Interestingly, *PR-1* mRNA accumulation was elevated in the *nim1-5* mutant, but only mildly induced following chemical treatment or *P. parasitica* infection. Based on both *PR-1* mRNA accumulation and fungal infection, the mutants fall into three classes: severely compromised alleles (*nim1-4* and *nim1-6*); moderately compromised alleles (*nim1-1*, *nim1-2*, and *nim1-3*); and a weakly compromised allele (*nim1-5*).

Fine Structure Mapping of the *nim1* Mutation

To determine a rough map position for *NIM1*, 74 F₂ *nim* phenotype plants from a cross between *nim1-1* (Ws-0) and Landsberg *erecta* (Ler) were identified for their susceptibility to *P. parasitica* and lack of accumulation of *PR-1* mRNA following INA treatment. After testing a number of simple sequence length polymorphism (SSLP) markers (Bell and Ecker 1994), *nim1* was found to lie about 8.2 centimorgans (cM) from nga128 and 8.2 cM from nga111 on the lower arm of chromosome 1. In subsequent analysis, *nim1-1* was found to lie between nga111 and about 4 cM from the SSLP marker ATHGENEA.

For fine structure mapping, 1138 *nim* plants from an F₂ population derived from a cross between *nim1-1* and Ler DP23 were identified based on both their inability to accumulate *PR-1*

mRNA and their ability to support fungal growth following INA treatment. DNA was extracted from these plants and scored for zygosity at both ATHGENEA and *nga111*. As shown in Figures 5A-5D, 93 recombinant chromosomes were identified between ATHGENEA and *nim1*, giving a genetic distance of approximately 4.1 cM (93 of 2276), and 239 recombinant chromosomes were identified between *nga111* and *nim1*, indicating a genetic distance of about 10.5 cM (239 of 2276). Informative recombinants in the ATHGENEA to *nga111* interval were further analyzed using amplified fragment length polymorphism (AFLP) analysis (Vos et al., 1995).

Initially, 10 AFLP markers between ATHGENEA and *nga111* were identified and these were used to construct a low resolution map of the region (Figure 5A). The AFLP markers W84.2 (1 cM from *nim1*) and W85.1 (0.6 cM from *nim1*) were used to isolate yeast artificial chromosome (YAC) clones from the CIC (for Centre d'Etude du Polymorphisme Humain, INRA and CNRS) library (Creusot et al., 1995). Two YAC clones, CIC12H07 and CIC12F04, were identified with W84.2 and two YAC clones CIC7E03 and CIC10G07 (data not shown) were identified with the W85.1 marker. However, it was determined that there was a gap between the two sets of flanking YAC clones. From this point, bacterial artificial chromosome (BAC) and P1 clones that overlapped CIC12H07 and CIC12F04 were isolated and mapped, and three sequential walking steps were then carried out extending the BAC/P1 contig toward *NIM1* (Liu et al., 1995; Chio et al., 1995). At various times during the walk, new AFLPs were developed that were specific for BAC or P1 clones, and these were used to determine whether the *NIM1* gene had been crossed. It was determined that *NIM1* had been crossed when BAC and P1 clones were isolated that gave rise to both AFLP markers L84.6a and L84.8. The AFLP marker L84.6a found on P1 clones P1-18, P1-17, and P1-21 identified three recombinants and L84.8 found on P1 clones P1-20, P1-22, P1-23, and P1-24 and BAC clones, BAC-04, BAC-05, and BAC-06 identified one recombinant. Because these clones overlap to form a large contig (>100 kb), and include AFLP markers that flank *nim1*, the gene was located on the contig. The BAC and P1 clones that comprised the contig were used to generate eight additional AFLP markers, which showed that *nim1* was located between L84.Y1 and L84.8, representing a gap of about 0.09 cM.

A cosmid library was constructed in the *Agrobacterium*-compatible T-DNA cosmid vector pCLD04541 using DNA from BAC-06, BAC-04, and P1-18. A cosmid contig was developed using AFLP markers derived from these clones. Physical mapping showed that the physical

distance between L84.Y1 and L84.8 was greater than 90 kb, giving a genetic to physical distance of roughly 1 megabase per cM. To facilitate the later identification of the *NIM1* gene, the DNA sequence of BAC-04 was determined.

5

Isolation of the *NIM1* Gene

To identify which cosmids contained the *NIM1* gene, the 12 cosmids listed in Table 4 of the Examples were transformed into *nim1-1*, and transformants were evaluated for their ability to complement the mutant phenotype. Cosmids D5, E1, and D7 were all found to complement *nim1-1*, as determined by the ability of the transformants to accumulate *PR-1* mRNA following INA treatment. The ends of these cosmids were sequenced and found to be located on the DNA sequence of BAC-04. There were 9,918 base pairs in the DNA region shared by D7 and D5 that contained the *NIM1* gene. As shown in Figure 5D, four putative gene regions were identified in this 10-kb sequence. Region 1 could potentially encode a protein of 19,105 D, region 3 could encode a protein of 44,554 D, and region 4 could encode a protein of 52,797 D. Region 2 had four open reading frames of various sizes located close together, suggesting a gene with three introns. Analysis using the NetPlantGene program (Hebsgaard et al., 1996) indicated a high probability that the open reading frames could be spliced together to form a large open reading frame encoding a protein of 66,039 D.

To ascertain which gene region contained the *NIM1* gene, gel blots containing RNA isolated from leaf tissue of Ws-0 and the different *nim1* mutants following either water or chemical treatment were probed with DNA derived from each of the four gene regions. In these experiments, care was taken to label probes to high specific activity and autoradiographs were exposed for more than 1 week. In our past experience, these conditions would identify RNA at concentrations of about one copy per cell. The only gene region that produced detectable RNA was gene region 2. As shown in Figure 7, the mRNA identified by the gene region 2 probe was induced by BTH treatment of wild-type plants, but not in any of the mutants. Furthermore, RNA accumulation was elevated in all of the plants following *P. parasitica* infection, indicating that this particular gene is induced following pathogen infection.

To further establish the gene region encoding *NIM1*, the DNA sequence from each of the four gene regions was determined for each of the *nim1* alleles and compared with the corresponding gene region from Ws-0. No mutations were detected between Ws-0 and the mutant alleles in either gene regions 3 or 4 and only a single change was found in gene region 1 in the *nim1-6* mutant.

However, a single base pair mutation was found in each of the alleles relative to Ws-0 for region 2. The DNA sequence of gene region 2 is shown in Figure 6. As shown in Table 5 and Figure 6, in *nim1-1*, a single adenosine is inserted at position 3579 that causes a frameshift resulting in a change in seven amino acids and a deletion of 349 amino acids. In *nim1-2*, there is a cytidine-to-thymidine transition at position 3763 that changes a histidine to a tyrosine. In *nim1-3*, a single adenosine is deleted at position 3301 causing a frameshift that altered 10 amino acids and deleted 412 from the predicted protein. Interestingly, both *nim1-4* and *nim1-5* have a guanosine-to-adenosine transition at position 4160 changing an arginine to a lysine, and in *nim1-6*, there is a cytosine-to-thymidine transition resulting in a stop codon causing the deletion of 255 amino acids from the predicted protein. Although the mutation in *nim1-4* and *nim1-5* alters the consensus donor splice site for the mRNA, RT-PCR analysis indicates that this mutation does not lead to an alteration of mRNA splicing (data not shown).

NIM1 Homologues

The gene region 2 DNA sequence was used in a Blast search (Altschul et al., 1990) and identified an exact match with the Arabidopsis expressed sequence tag (EST) T22612 and significant matches to the rice ESTs S2556, S2861, S3060 and S3481. A DNA probe covering base pairs 2081 to 3266 was used to screen an Arabidopsis cDNA library, and 14 clones were isolated that correspond to gene region 2. From the cDNA sequence, we could confirm the placement of the exon/intron borders shown in Figure 6. Rapid amplification of cDNA ends by polymerase chain reaction (RACE) was carried out using RNA from INA-treated Ws-0 plants and the likely transcriptional start site was determined to be the A at position 2588 in Figure 6.

Using the *NIM1* cDNA as a probe, homologs of Arabidopsis *NIM1* can be identified and isolated through screening genomic or cDNA libraries from different plants such as, but not limited to following crop plants: rice, wheat, barley, rye, corn, potato, carrot, sweet potato, sugar beet, bean,

pea, chicory, lettuce, cabbage, cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tobacco, tomato, sorghum and sugarcane. Standard techniques for accomplishing this include hybridization screening of plated DNA libraries (either
5 plaques or colonies; see, *e.g.* Sambrook *et al.*, Molecular Cloning, eds., Cold Spring Harbor Laboratory Press. (1989)) and amplification by PCR using oligonucleotide primers (see, *e.g.* Innis *et al.*, PCR Protocols, a Guide to Methods and Applications eds., Academic Press (1990)).

Homologues identified are genetically engineered into the expression vectors listed below and
10 transformed into the above listed crops. Transformants are evaluated for enhanced disease resistance using relevant pathogens of the crop plant being tested.

For example, *NIM1* homologs in the genomes of cucumber, tomato, tobacco, maize, wheat and barley have been detected by DNA blot analysis. Genomic DNA was isolated from cucumber, tomato, tobacco, maize, wheat and barley, restriction digested with the enzymes BamHI, HindIII, XbaI, or SalI, electrophoretically separated on 0.8% agarose gels and transferred to nylon
15 membrane by capillary blotting. Following UV-crosslinking to affix the DNA, the membrane was hybridized under low stringency conditions [(1%BSA; 520mM NaPO₄, pH7.2; 7% lauryl sulfate, sodium salt; 1mM EDTA; 250 mM sodium chloride) at 55°C for 18-24h] with ³²P-radiolabelled *Arabidopsis thaliana* *NIM1* cDNA. Following hybridization the blots were washed under low
20 stringency conditions [6XSSC for 15 min. (X3) 3XSSC for 15 min. (X1) at 55°C; 1XSSC is 0.15M NaCl, 15mM Na-citrate (pH7.0)] and exposed to X-ray film to visualize bands that correspond to *NIM1*.

In addition, expressed sequence tags (EST) identified with similarity to the *NIM1* gene such as the rice EST's described above can also be used to isolate homologues. The rice EST's may be
25 especially useful for isolation of *NIM1* homologues from other monocots.

Homologues may also be obtained by PCR. In this method, comparisons are made between known homologues (*e.g.*, rice and *Arabidopsis*). Regions of high amino acid and DNA similarity or identity are then used to make PCR primers. Once a suitable region is identified, primers for that region are made with a diversity of substitutions in the 3rd codon position. The PCR reaction is

performed from cDNA or genomic DNA under a variety of standard conditions. When a band is apparent, it is cloned and/or sequences to determine if it is a *NIM1* homologue.

High-Level Expression of *NIM1* Confers Disease Resistance In Plants

The present invention also concerns the production of transgenic plants that express higher-than-wild-type levels of the *NIM1* gene, or functional variants and mutants thereof, and thereby have broad spectrum disease resistance. In a preferred embodiment of the invention, the expression of the *NIM1* gene is at a level which is at least two-fold above the expression level of the *NIM1* gene in wild-type plants and is preferably tenfold above the wild-type expression level. High-level expression of the *NIM1* gene mimics the effects of inducer compounds in that it gives rise to plants with a constitutive immunity (CIM) phenotype.

Several methods are described for producing plants that overexpress the *NIM1* gene and thereby have a CIM phenotype. A first method is selecting transformed plants that have high-level expression of *NIM1* and therefore a CIM phenotype due to insertion site effect. Table 6 shows the results of testing of various transformants for resistance to fungal infection. As can be seen from this table, a number of transformants showed less than normal fungal growth and several showed no visible fungal growth at all. RNA was prepared from collected samples and analyzed as described in Delaney et al, 1995. Blots were hybridized to the *Arabidopsis* gene probe PR-1 (Uknes et al, 1992). Three lines showed early induction of PR-1 gene expression in that PR-1 mRNA was evident by 24 or 48 hours following fungal treatment. These three lines also demonstrated resistance to fungal infection.

In addition, methods are described for constructing plant transformation vectors comprising a constitutive plant-active promoter, such as the CaMV 35S promoter, operatively linked to a coding region that encodes an active NIM1 protein. High levels of the active NIM1 protein produce the same disease-resistance effect as chemical induction with inducing chemicals such as BTH, INA, and SA.

The overexpression of the *NIM1* gene in plants results in immunity to a wide array of plant pathogens, which include, but are not limited to viruses or viroids, e.g. tobacco or cucumber mosaic virus, ringspot virus or necrosis virus, pelargonium leaf curl virus, red clover mottle virus, tomato

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bushy stunt virus, and like viruses; fungi, e.g. *Phytophthora parasitica* and *Peronospora tabacina*;
bacteria, e.g. *Pseudomonas syringae* and *Pseudomonas tabaci*; insects such as aphids, e.g. *Myzus*
persicae; and lepidoptera, e.g., *Heliothus spp.*; and nematodes, e.g., *Meloidogyne incognita*. The
vectors and methods of the invention are useful against a number of disease organisms of maize
5 including but not limited to downy mildews such as *Sclerophthora macrospora*, *Sclerophthora*
rayissiae, *Sclerospora graminicola*, *Peronosclerospora sorghi*, *Peronosclerospora philippinensis*,
Peronosclerospora sacchari and *Peronosclerospora maydis*; rusts such as *Puccinia sorphi*,
Puccinia polysora and *Physopella zae*; other fungi such as *Cercospora zae-maydis*,
Colletotrichum graminicola, *Fusarium monoliforme*, *Gibberella zae*, *Exserohilum turcicum*,
10 *Kabatiellu zae*, *Erysiphe graminis*, *Septoria* and *Bipolaris maydis*; and bacteria such as *Erwinia*
stewartii.

The methods of the present invention can be utilized to confer disease resistance to a wide
variety of plants, including gymnosperms, monocots, and dicots. Although disease resistance can
be conferred upon any plants falling within these broad classes, it is particularly useful in
15 agronomically important crop plants, such as rice, wheat, barley, rye, corn, potato, carrot, sweet
potato, sugar beet, bean, pea, chicory, lettuce, cabbage, cauliflower, broccoli, turnip, radish,
spinach, asparagus, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini,
cucumber, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape,
raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tobacco, tomato,
20 sorghum and sugarcane. Transformed cells can be regenerated into whole plants such that the gene
imparts disease resistance to the intact transgenic plants. The expression system can be modified so
that the disease resistance gene is continuously or constitutively expressed.

Recombinant DNA Technology

25 The *NIMI* DNA molecule or gene fragment conferring disease resistance to plants by
allowing induction of SAR gene expression can be incorporated in plant or bacterial cells using
conventional recombinant DNA technology. Generally, this involves inserting the DNA molecule
comprised within SEQ ID NO:1 or a functional variant thereof into an expression system to which
30 the DNA molecule is heterologous (i.e., not normally present). The heterologous DNA molecule is

inserted into the expression system or vector in proper orientation and correct reading frame. The vector contains the necessary elements for the transcription and translation of the inserted protein-coding sequences. A large number of vector systems known in the art can be used, such as plasmids, bacteriophage viruses and other modified viruses. Suitable vectors include, but are not limited to, viral vectors such as lambda vector systems lgt11, lgt10 and Charon 4; plasmid vectors such as pBI121, pBR322, pACYC177, pACYC184, pAR series, pKK223-3, pUC8, pUC9, pUC18, pUC19, pLG339, pRK290, pKC37, pKC101, pCDNAlI; and other similar systems. The *NIM1* coding sequence described herein can be cloned into the vector using standard cloning procedures in the art, as described by Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory, Cold Spring Harbor, New York (1982).

In order to obtain efficient expression of the gene or gene fragment of the present invention, a promoter which will result in a sufficient expression level or constitutive expression must be present in the expression vector. RNA polymerase normally binds to the promoter and initiates transcription of a gene. Promoters vary in their strength, i.e., ability to promote transcription. Depending upon the host cell system utilized, any one of a number of suitable promoters can be used. The components of the expression cassette may be modified to increase expression. For example, truncated sequences, nucleotide substitutions or other modifications may be employed. Plant cells transformed with such modified expression systems, then, exhibit overexpression or constitutive expression of genes necessary for activation of SAR.

A. Construction of Plant Transformation Vectors

Numerous transformation vectors are available for plant transformation, and the genes of this invention can be used in conjunction with any such vectors. The selection of vector will depend upon the preferred transformation technique and the target species for transformation. For certain target species, different antibiotic or herbicide selection markers may be preferred. Selection markers used routinely in transformation include the *nptII* gene which confers resistance to kanamycin and related antibiotics (Messing & Vierra. Gene 19: 259-268 (1982); Bevan et al., Nature 304:184-187 (1983)), the *bar* gene, which confers resistance to the herbicide phosphinothricin (White et al., Nucl. Acids Res 18: 1062 (1990), Spencer et al. Theor. Appl. Genet 79: 625-631 (1990)), the *hph* gene, which confers resistance to the antibiotic hygromycin

(Blochinger & Diggelmann, Mol Cell Biol 4: 2929-2931), and the *dhfr* gene, which confers resistance to methatrexate (Bourouis et al., EMBO J. 2(7): 1099-1104 (1983)), and the EPSPS gene, which confers resistance to glyphosate (U.S. Patent Nos. 4,940,935 and 5,188,642).

1. Vectors Suitable for Agrobacterium Transformation

Many vectors are available for transformation using *Agrobacterium tumefaciens*. These typically carry at least one T-DNA border sequence and include vectors such as pBIN19 (Bevan, Nucl. Acids Res. (1984)) and pXYZ. Below, the construction of two typical vectors is described.

a. pCIB200 and pCIB2001:

The binary vectors pCIB200 and pCIB2001 are used for the construction of recombinant vectors for use with *Agrobacterium* and are constructed in the following manner. pTJS75kan is created by *NarI* digestion of pTJS75 (Schmidhauser & Helinski, J. Bacteriol. 164: 446-455 (1985)) allowing excision of the tetracycline-resistance gene, followed by insertion of an *AccI* fragment from pUC4K carrying an NPTII (Messing & Vierra, Gene 19: 259-268 (1982); Bevan et al., Nature 304: 184-187 (1983); McBride et al., Plant Molecular Biology 14: 266-276 (1990)). *XhoI* linkers are ligated to the *EcoRV* fragment of PCIB7 which contains the left and right T-DNA borders, a plant selectable *nos/nptII* chimeric gene and the pUC polylinker (Rothstein et al., Gene 53: 153-161 (1987)), and the *XhoI*-digested fragment are cloned into *Sall*-digested pTJS75kan to create pCIB200 (see also EP 0 332 104, example 19). pCIB200 contains the following unique polylinker restriction sites: *EcoRI*, *SstI*, *KpnI*, *BglII*, *XbaI*, and *Sall*. pCIB2001 is a derivative of pCIB200 created by the insertion into the polylinker of additional restriction sites. Unique restriction sites in the polylinker of pCIB2001 are *EcoRI*, *SstI*, *KpnI*, *BglII*, *XbaI*, *Sall*, *MluI*, *BclI*, *AvrII*, *ApaI*, *HpaI*, and *StuI*. pCIB2001, in addition to containing these unique restriction sites also has plant and bacterial kanamycin selection, left and right T-DNA borders for *Agrobacterium*-mediated transformation, the RK2-derived *trfA* function for mobilization between *E. coli* and other hosts, and the *OriT* and *OriV* functions also from RK2. The pCIB2001 polylinker is suitable for the cloning of plant expression cassettes containing their own regulatory signals.

b. pCIB10 and Hygromycin Selection Derivatives thereof:

The binary vector pCIB10 contains a gene encoding kanamycin resistance for selection in plants and T-DNA right and left border sequences and incorporates sequences from the wide host-range plasmid pRK252 allowing it to replicate in both *E. coli* and *Agrobacterium*. Its construction is described by Rothstein *et al.* (Gene 53: 153-161 (1987)). Various derivatives of pCIB10 are constructed which incorporate the gene for hygromycin B phosphotransferase described by Gritz *et al.* (Gene 25: 179-188 (1983)). These derivatives enable selection of transgenic plant cells on hygromycin only (pCIB743), or hygromycin and kanamycin (pCIB715, pCIB717).

2. Vectors Suitable for non-*Agrobacterium* Transformation

Transformation without the use of *Agrobacterium tumefaciens* circumvents the requirement for T-DNA sequences in the chosen transformation vector and consequently vectors lacking these sequences can be utilized in addition to vectors such as the ones described above which contain T-DNA sequences. Transformation techniques which do not rely on *Agrobacterium* include transformation via particle bombardment, protoplast uptake (*e.g.* PEG and electroporation) and microinjection. The choice of vector depends largely on the preferred selection for the species being transformed.

a. pCIB3064:

pCIB3064 is a pUC-derived vector suitable for direct gene transfer techniques in combination with selection by the herbicide basta (or pho*Sph*Inothricin). The plasmid pCIB246 comprises the CaMV 35S promoter in operational fusion to the *E. coli* GUS gene and the CaMV 35S transcriptional terminator and is described in the PCT published application WO 93/07278. The 35S promoter of this vector contains two ATG sequences 5' of the start site. These sites are mutated using standard PCR techniques in such a way as to remove the ATGs and generate the restriction sites *SspI* and *PvuII*. The new restriction sites are 96 and 37 bp away from the unique *Sall* site and 101 and 42 bp away from the actual start site. The resultant derivative of pCIB246 is designated pCIB3025. The GUS gene is then excised from pCIB3025 by digestion with *Sall* and *SacI*, the termini rendered blunt and religated to generate plasmid pCIB3060. The plasmid pJIT82 is obtained from the John Innes Centre, Norwich and the a 400 bp *SmaI* fragment containing the

bar gene from *Streptomyces viridochromogenes* is excised and inserted into the *HpaI* site of pCIB3060 (Thompson *et al.* EMBO J 6: 2519-2523 (1987)). This generated pCIB3064, which comprises the *bar* gene under the control of the CaMV 35S promoter and terminator for herbicide selection, a gene for ampicillin resistance (for selection in *E. coli*) and a polylinker with the unique sites *SphI*, *PstI*, *HindIII*, and *BamHI*. This vector is suitable for the cloning of plant expression cassettes containing their own regulatory signals.

b. pSOG19 and pSOG35:

pSOG35 is a transformation vector which utilizes the *E. coli* gene dihydrofolate reductase (DFR) as a selectable marker conferring resistance to methotrexate. PCR is used to amplify the 35S promoter (-800 bp), intron 6 from the maize *Adh1* gene (-550 bp) and 18 bp of the GUS untranslated leader sequence from pSOG10. A 250-bp fragment encoding the *E. coli* dihydrofolate reductase type II gene is also amplified by PCR and these two PCR fragments are assembled with a *SacI-PstI* fragment from pB1221 (Clontech) which comprises the pUC19 vector backbone and the nopaline synthase terminator. Assembly of these fragments generates pSOG19 which contains the 35S promoter in fusion with the intron 6 sequence, the GUS leader, the DHFR gene and the nopaline synthase terminator. Replacement of the GUS leader in pSOG19 with the leader sequence from Maize Chlorotic Mottle Virus (MCMV) generates the vector pSOG35. pSOG19 and pSOG35 carry the pUC gene for ampicillin resistance and have *HindIII*, *SphI*, *PstI* and *EcoRI* sites available for the cloning of foreign substances.

B. Requirements for Construction of Plant Expression Cassettes

Gene sequences intended for expression in transgenic plants are first assembled in expression cassettes behind a suitable high expression level promoter and upstream of a suitable transcription terminator. These expression cassettes can then be easily transferred to the plant transformation vectors described above.

1. Promoter Selection

The selection of the promoter used in expression cassettes will determine the spatial and temporal expression pattern of the transgene in the transgenic plant. Selected promoters will express transgenes in specific cell types (such as leaf epidermal cells, mesophyll cells, root cortex cells) or in specific tissues or organs (roots, leaves or flowers, for example) and the selection will reflect the desired location of accumulation of the *NIM1* gene product. Alternatively, the selected promoter may drive expression of the gene under a light-induced or other temporally regulated promoter.

a. Constitutive Expression, the CaMV 35S Promoter:

Construction of the plasmid pCGN1761 is described in the published patent application EP 0 392 225 (example 23) which is hereby incorporated by reference. pCGN1761 contains the "double" 35S promoter and the *tml* transcriptional terminator with a unique *EcoRI* site between the promoter and the terminator and has a pUC-type backbone. A derivative of pCGN1761 is constructed which has a modified polylinker which includes *NotI* and *XhoI* sites in addition to the existing *EcoRI* site. This derivative is designated pCGN1761ENX. pCGN1761ENX is useful for the cloning of cDNA sequences or gene sequences (including microbial ORF sequences) within its polylinker for the purpose of their expression under the control of the 35S promoter in transgenic plants. The entire 35S promoter-gene sequence-*tml* terminator cassette of such a construction can be excised by *HindIII*, *SphI*, *Sall*, and *XbaI* sites 5' to the promoter and *XbaI*, *BamHI* and *BglI* sites 3' to the terminator for transfer to transformation vectors such as those described above. Furthermore, the double 35S promoter fragment can be removed by 5' excision with *HindIII*, *SphI*, *Sall*, *XbaI*, or *PstI*, and 3' excision with any of the polylinker restriction sites (*EcoRI*, *NotI* or *XhoI*) for replacement with another promoter.

b. Modification of pCGN1761ENX by Optimization of the Translational Initiation Site:

For any of the constructions described herein, modifications around the cloning sites can be made by the introduction of sequences which may enhance translation. This is particularly useful when overexpression is desired.

pCGN1761ENX is cleaved with *SphI*, treated with T4 DNA polymerase and religated, thus destroying the *SphI* site located 5' to the double 35S promoter. This generates vector pCGN1761ENX/*Sph*-. pCGN1761ENX/*Sph*- is cleaved with *EcoRI*, and ligated to an annealed molecular adaptor of the sequence 5'-AATTCTAAAGCATGCCGATCGG-3'/5'-

5 AATTCCGATCGGCATGCTTTA-3' (SEQ ID NO's: 12 and 13). This generates the vector pCGNSENX, which incorporates the *quasi*-optimized plant translational initiation sequence TAAA-C adjacent to the ATG which is itself part of an *SphI* site which is suitable for cloning heterologous genes at their initiating methionine. Downstream of the *SphI* site, the *EcoRI*, *NotI*, and *XhoI* sites are retained.

10 An alternative vector is constructed which utilizes an *NcoI* site at the initiating ATG. This vector, designated pCGN1761NENX is made by inserting an annealed molecular adaptor of the sequence 5'-AATTCTAAACCATGGCGATCGG-3'/5'-AATTCCGATCGCCATGGTTTA-3' (SEQ ID NO's: 14 and 15) at the pCGN1761ENX *EcoRI* site. Thus the vector includes the *quasi*-optimized sequence TAAACC adjacent to the initiating ATG which is within the *NcoI* site. Downstream sites are *EcoRI*, *NotI*, and *XhoI*. Prior to this manipulation, however, the two *NcoI* sites in the pCGN1761ENX vector (at upstream positions of the 5' 35S promoter unit) are destroyed using similar techniques to those described above for *SphI* or alternatively using "inside-outside" PCR. Innes *et al.* PCR Protocols: A guide to methods and applications. Academic Press, New York (1990). This manipulation can be assayed for any possible detrimental effect on
20 expression by insertion of any plant cDNA or reporter gene sequence into the cloning site followed by routine expression analysis in plants.

c. Expression under a Chemically/Pathogen Regulatable Promoter:

25 The double 35S promoter in pCGN1761ENX may be replaced with any other promoter of choice which will result in suitably high expression levels. By way of example, a chemically regulated PR-1 promoter, which is described in U.S. Patent No. 5,614,395, which is hereby incorporated by reference in its entirety, may replace the double 35S promoter. The promoter of choice is preferably excised from its source by restriction enzymes, but can alternatively be PCR-amplified using primers which carry appropriate terminal restriction sites. Should PCR-
30 amplification be undertaken, then the promoter should be re-sequenced to check for amplification

errors after the cloning of the amplified promoter in the target vector. The chemically/pathogen regulatable tobacco PR-1a promoter is cleaved from plasmid pCIB1004 (see EP 0 332 104, example 21 for construction which is hereby incorporated by reference) and transferred to plasmid pCGN1761ENX (Uknes et al. 1992). pCIB1004 is cleaved with *NcoI* and the resultant 3' overhang of the linearized fragment is rendered blunt by treatment with T4 DNA polymerase. The fragment is then cleaved with *HindIII* and the resultant PR-1a-promoter-containing fragment is gel purified and cloned into pCGN1761ENX from which the double 35S promoter has been removed. This is done by cleavage with *XhoI* and blunting with T4 polymerase, followed by cleavage with *HindIII* and isolation of the larger vector-terminator containing fragment into which the pCIB1004 promoter fragment is cloned. This generates a pCGN1761ENX derivative with the PR-1a promoter and the *tml* terminator and an intervening polylinker with unique *EcoRI* and *NotI* sites. Selected *NIM1* genes can be inserted into this vector, and the fusion products (*i.e.* promoter-gene-terminator) can subsequently be transferred to any selected transformation vector, including those described in this application.

Various chemical regulators may be employed to induce expression of the *NIM1* coding sequence in the plants transformed according to the present invention. In the context of the instant disclosure, "chemical regulators" include chemicals known to be inducers for the PR-1 promoter in plants, or close derivatives thereof. A preferred group of regulators for the PR-1 promoter is based on the benzo-1,2,3-thiadiazole (BTH) structure and includes, but is not limited to, the following types of compounds: benzo-1,2,3-thiadiazolecarboxylic acid, benzo-1,2,3-thiadiazolethiocarboxylic acid, cyanobenzo-1,2,3-thiadiazole, benzo-1,2,3-thiadiazolecarboxylic acid amide, benzo-1,2,3-thiadiazolecarboxylic acid hydrazide, benzo-1,2,3-thiadiazole-7-carboxylic acid, benzo-1,2,3-thiadiazole-7-thiocarboxylic acid, 7-cyanobenzo-1,2,3-thiadiazole, benzo-1,2,3-thiadiazolecarboxylate in which the alkyl group contains one to six carbon atoms, methyl benzo-1,2,3-thiadiazole-7-carboxylate, n-propyl benzo-1,2,3-thiadiazole-7-carboxylate, benzyl benzo-1,2,3-thiadiazole-7-carboxylate, benzo-1,2,3-thiadiazole-7-carboxylic acid sec-butylhydrazide, and suitable derivatives thereof. Other chemical inducers may include, for example, benzoic acid, salicylic acid (SA), polyacrylic acid and substituted derivatives thereof; suitable substituents include lower alkyl, lower alkoxy, lower alkylthio, and halogen. Still another group of regulators for the chemically inducible DNA sequences of this invention is based on the pyridine carboxylic acid

structure, such as the isonicotinic acid structure and preferably the haloisonicotinic acid structure. Preferred are dichloroisonicotinic acids and derivatives thereof, for example the lower alkyl esters. Suitable members of this class of regulator compounds are, for example, 2,6-dichloroisonicotinic acid (INA), and the lower alkyl esters thereof, especially the methyl ester.

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d. Constitutive Expression, the Actin Promoter:

Several isoforms of actin are known to be expressed in most cell types and consequently the actin promoter is a good choice for a constitutive promoter. In particular, the promoter from the rice *Act1* gene has been cloned and characterized (McElroy *et al.* Plant Cell 2: 163-171 (1990)). A 1.3kb fragment of the promoter was found to contain all the regulatory elements required for expression in rice protoplasts. Furthermore, numerous expression vectors based on the *Act1* promoter have been constructed specifically for use in monocotyledons (McElroy *et al.* Mol. Gen. Genet. 231: 150-160 (1991)). These incorporate the *Act1*-intron 1, *Adh1* 5' flanking sequence and *Adh1*-intron 1 (from the maize alcohol dehydrogenase gene) and sequence from the CaMV 35S promoter. Vectors showing highest expression were fusions of 35S and *Act1* intron or the *Act1* 5' flanking sequence and the *Act1* intron. Optimization of sequences around the initiating ATG (of the GUS reporter gene) also enhanced expression. The promoter expression cassettes described by McElroy *et al.* (Mol. Gen. Genet. 231: 150-160 (1991)) can be easily modified for the expression of cellulase genes and are particularly suitable for use in monocotyledonous hosts. For example, promoter-containing fragments is removed from the McElroy constructions and used to replace the double 35S promoter in pCGN1761ENX, which is then available for the insertion of specific gene sequences. The fusion genes thus constructed can then be transferred to appropriate transformation vectors. In a separate report the rice *Act1* promoter with its first intron has also been found to direct high expression in cultured barley cells (Chibbar *et al.* Plant Cell Rep. 12: 506-509 (1993)).

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e. Constitutive Expression, the Ubiquitin Promoter:

Ubiquitin is another gene product known to accumulate in many cell types and its promoter has been cloned from several species for use in transgenic plants (*e.g.* sunflower - Binet *et al.* Plant Science 79: 87-94 (1991) and maize - Christensen *et al.* Plant Molec. Biol. 12: 619-632 (1989)).

30 The maize ubiquitin promoter has been developed in transgenic monocot systems and its sequence

and vectors constructed for monocot transformation are disclosed in the patent publication EP 0 342 926 (to Lubrizol) which is herein incorporated by reference. Taylor *et al.* (Plant Cell Rep. 12: 491-495 (1993)) describe a vector (pAHC25) which comprises the maize ubiquitin promoter and first intron and its high activity in cell suspensions of numerous monocotyledons when introduced via microprojectile bombardment. The ubiquitin promoter is suitable for the expression of cellulase genes in transgenic plants, especially monocotyledons. Suitable vectors are derivatives of pAHC25 or any of the transformation vectors described in this application, modified by the introduction of the appropriate ubiquitin promoter and/or intron sequences.

f. Root Specific Expression:

Another pattern of expression for the *NIM1* gene of the instant invention is root expression. A suitable root promoter is described by de Framond (FEBS 290: 103-106 (1991)) and also in the published patent application EP 0 452 269 (to Ciba-Geigy) which is herein incorporated by reference. This promoter is transferred to a suitable vector such as pCGN1761ENX for the insertion of a cellulase gene and subsequent transfer of the entire promoter-gene-terminator cassette to a transformation vector of interest.

g. Wound-Inducible Promoters:

Wound-inducible promoters may also be suitable for expression of *NIM1* genes of the invention. Numerous such promoters have been described (*e.g.* Xu *et al.* Plant Molec. Biol. 22: 573-588 (1993), Logemann *et al.* Plant Cell 1: 151-158 (1989), Rohrmeier & Lehle, Plant Molec. Biol. 22: 783-792 (1993), Firek *et al.* Plant Molec. Biol. 22: 129-142 (1993), Warner *et al.* Plant J. 3: 191-201 (1993)) and all are suitable for use with the instant invention. Logemann *et al.* describe the 5' upstream sequences of the dicotyledonous potato *wun1* gene. Xu *et al.* show that a wound-inducible promoter from the dicotyledon potato (*pin2*) is active in the monocotyledon rice. Further, Rohrmeier & Lehle describe the cloning of the maize *Wip1* cDNA which is wound induced and which can be used to isolate the cognate promoter using standard techniques. Similar, Firek *et al.* and Warner *et al.* have described a wound-induced gene from the monocotyledon *Asparagus officinalis* which is expressed at local wound and pathogen invasion sites. Using cloning

techniques well known in the art, these promoters can be transferred to suitable vectors, fused to the *NIM1* genes of this invention, and used to express these genes at the sites of plant wounding.

h. Pith-Preferred Expression:

Patent Application WO 93/07278 (to Ciba-Geigy) which is herein incorporated by reference describes the isolation of the maize *trpA* gene which is preferentially expressed in pith cells. The gene sequence and promoter extending up to -1726 bp from the start of transcription are presented. Using standard molecular biological techniques, this promoter, or parts thereof, can be transferred to a vector such as pCGN1761 where it can replace the 35S promoter and be used to drive the expression of a foreign gene in a pith-preferred manner. In fact, fragments containing the pith-preferred promoter or parts thereof can be transferred to any vector and modified for utility in transgenic plants.

i. Leaf-Specific Expression:

A maize gene encoding phosphoenol carboxylase (PEPC) has been described by Hudspeth & Grula (Plant Molec Biol 12: 579-589 (1989)). Using standard molecular biological techniques the promoter for this gene can be used to drive the expression of any gene in a leaf-specific manner in transgenic plants.

j. Expression with Chloroplast Targeting:

Chen & Jagendorf (J. Biol. Chem. 268: 2363-2367 (1993) have described the successful use of a chloroplast transit peptide for import of a heterologous transgene. This peptide used is the transit peptide from the *rbcS* gene from *Nicotiana plumbaginifolia* (Poulsen *et al.* Mol. Gen. Genet. 205: 193-200 (1986)). Using the restriction enzymes *DraI* and *SphI*. pr *Tsp509I* and *SphI* the DNA sequence encoding this transit peptide can be excised from the plasmid prbcS-8B and manipulated for use with any of the constructions described above. The *DraI-SphI* fragment extends from -58 relative to the initiating *rbcS* ATG to, and including, the first amino acid (also a methionine) of the mature peptide immediately after the import cleavage site, whereas the *Tsp509I-SphI* fragment extends from -8 relative to the initiating *rbcS* ATG to, and including, the first amino acid of the mature peptide.

Thus, these fragments can be appropriately inserted into the polylinker of any chosen expression cassette generating a transcriptional fusion to the untranslated leader of the chosen promoter (*e.g.* 35S, PR-1a, actin, ubiquitin *etc.*), while enabling the insertion of a *NIM1* gene in correct fusion downstream of the transit peptide. Constructions of this kind are routine in the art.

For example, whereas the *DraI* end is already blunt, the 5' *Tsp509I* site may be rendered blunt by T4 polymerase treatment, or may alternatively be ligated to a linker or adaptor sequence to facilitate its fusion to the chosen promoter. The 3' *SphI* site may be maintained as such, or may alternatively be ligated to adaptor or linker sequences to facilitate its insertion into the chosen vector in such a way as to make available appropriate restriction sites for the subsequent insertion of a selected *NIM1* gene. Ideally the ATG of the *SphI* site is maintained and comprises the first ATG of the selected *NIM1* gene. Chen & Jagendorf provide consensus sequences for ideal cleavage for chloroplast import, and in each case a methionine is preferred at the first position of the mature protein. At subsequent positions there is more variation and the amino acid may not be so critical. In any case, fusion constructions can be assessed for efficiency of import *in vitro* using the methods described by Bartlett *et al.* (In: Edelman *et al.* (Eds.) *Methods in Chloroplast Molecular Biology*, Elsevier pp 1081-1091 (1982)) and Wasmann *et al.* (*Mol. Gen. Genet.* 205: 446-453 (1986)). Typically the best approach may be to generate fusions using the selected *NIM1* gene with no modifications at the amino terminus, and only to incorporate modifications when it is apparent that such fusions are not chloroplast imported at high efficiency, in which case modifications may be made in accordance with the established literature (Chen & Jagendorf; Wasman *et al.*; Ko & Ko, J. Biol. Chem 267: 13910-13916 (1992)).

A preferred vector is constructed by transferring the *DraI-SphI* transit peptide encoding fragment from prbcS-8B to the cloning vector pCGN1761ENX/Sph-. This plasmid is cleaved with *EcoRI* and the termini rendered blunt by treatment with T4 DNA polymerase. Plasmid prbcS-8B is cleaved with *SphI* and ligated to an annealed molecular adaptor of the sequence 5'-CCAGCTGGAATTCCG-3'/5'-CGGAATTCCAGCTGGCATG-3' (SEQ ID NO's: 16 and 17). The resultant product is 5'-terminally phosphorylated by treatment with T4 kinase. Subsequent cleavage with *DraI* releases the transit peptide encoding fragment which is ligated into the blunt-end ex-*EcoRI* sites of the modified vector described above. Clones oriented with the 5' end of the insert adjacent to the 3' end of the 35S promoter are identified by sequencing. These clones carry a

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DNA fusion of the 35S leader sequence to the *rbcs*-8A promoter-transit peptide sequence extending from -58 relative to the *rbcs* ATG to the ATG of the mature protein, and including in that region a unique *SphI* site, and a newly created *EcoRI* site, as well as the existing *NotI* and *XhoI* sites of pCGN1761ENX. This new vector is designated pCGN1761/CT. DNA sequences are transferred to pCGN1761/CT in frame by amplification using PCR techniques and incorporation of an *SphI*, *NSphI*, or *NlaIII* site at the amplified ATG, which following restriction enzyme cleavage with the appropriate enzyme is ligated into *SphI*-cleaved pCGN1761/CT. To facilitate construction, it may be required to change the second amino acid of the product of the cloned gene; however, in almost all cases the use of PCR together with standard site directed mutagenesis will enable the construction of any desired sequence around the cleavage site and first methionine of the mature protein.

A further preferred vector is constructed by replacing the double 35S promoter of pCGN1761ENX with the *BamHI*-*SphI* fragment of *prbcS*-8A which contains the full-length, light-regulated *rbcs*-8A promoter from -1038 (relative to the transcriptional start site) up to the first methionine of the mature protein. The modified pCGN1761 with the destroyed *SphI* is cleaved with *PstI* and *EcoRI* and treated with T4 DNA polymerase to render termini blunt. *prbcS*-8A is cleaved with *SphI* and ligated to the annealed molecular adaptor of the sequence described above. The resultant product is 5'-terminally phosphorylated by treatment with T4 kinase. Subsequent cleavage with *BamHI* releases the promoter-transit peptide containing fragment which is treated with T4 DNA polymerase to render the *BamHI* terminus blunt. The promoter-transit peptide fragment thus generated is cloned into the prepared pCGN1761ENX vector, generating a construction comprising the *rbcs*-8A promoter and transit peptide with an *SphI* site located at the cleavage site for insertion of heterologous genes. Further, downstream of the *SphI* site there are *EcoRI* (re-created), *NotI*, and *XhoI* cloning sites. This construction is designated pCGN1761*rbcs*/CT.

Similar manipulations can be undertaken to utilize other GS2 chloroplast transit peptide encoding sequences from other sources (monocotyledonous and dicotyledonous) and from other genes. In addition, similar procedures can be followed to achieve targeting to other subcellular compartments such as mitochondria.

2. Transcriptional Terminators

A variety of transcriptional terminators are available for use in expression cassettes. These are responsible for the termination of transcription beyond the transgene and its correct polyadenylation. Appropriate transcriptional terminators are those which are known to function in plants and include the CaMV 35S terminator, the *tml* terminator, the nopaline synthase terminator and the pea *rbcS* E9 terminator. These can be used in both monocotyledons and dicotyledons.

3. Sequences for the Enhancement or Regulation of Expression

Numerous sequences have been found to enhance gene expression from within the transcriptional unit and these sequences can be used in conjunction with the genes of this invention to increase their expression in transgenic plants.

Various intron sequences have been shown to enhance expression, particularly in monocotyledonous cells. For example, the introns of the maize *Adh1* gene have been found to significantly enhance the expression of the wild-type gene under its cognate promoter when introduced into maize cells. Intron 1 was found to be particularly effective and enhanced expression in fusion constructs with the chloramphenicol acetyltransferase gene (Callis *et al.*, Genes Develop. 1: 1183-1200 (1987)). In the same experimental system, the intron from the maize *bronze1* gene had a similar effect in enhancing expression. Intron sequences have been routinely incorporated into plant transformation vectors, typically within the non-translated leader.

A number of non-translated leader sequences derived from viruses are also known to enhance expression, and these are particularly effective in dicotyledonous cells. Specifically, leader sequences from Tobacco Mosaic Virus (TMV, the "W-sequence"), Maize Chlorotic Mottle Virus (MCMV), and Alfalfa Mosaic Virus (AMV) have been shown to be effective in enhancing expression (*e.g.* Gallie *et al.* Nucl. Acids Res. 15: 8693-8711 (1987); Skuzeski *et al.* Plant Molec. Biol. 15: 65-79 (1990)).

4. Targeting of the Gene Product Within the Cell

Various mechanisms for targeting gene products are known to exist in plants and the sequences controlling the functioning of these mechanisms have been characterized in some detail.

For example, the targeting of gene products to the chloroplast is controlled by a signal sequence

found at the amino terminal end of various proteins which is cleaved during chloroplast import to yield the mature protein (*e.g.* Comai *et al.* J. Biol. Chem. 263: 15104-15109 (1988)). These signal sequences can be fused to heterologous gene products to effect the import of heterologous products into the chloroplast (van den Broeck, *et al.* Nature 313: 358-363 (1985)). DNA encoding for appropriate signal sequences can be isolated from the 5' end of the cDNAs encoding the RUBISCO protein, the CAB protein, the EPSP synthase enzyme, the GS2 protein and many other proteins which are known to be chloroplast localized.

Other gene products are localized to other organelles such as the mitochondrion and the peroxisome (*e.g.* Unger *et al.* Plant Molec. Biol. 13: 411-418 (1989)). The cDNAs encoding these products can also be manipulated to effect the targeting of heterologous gene products to these organelles. Examples of such sequences are the nuclear-encoded ATPases and specific aspartate amino transferase isoforms for mitochondria. Targeting cellular protein bodies has been described by Rogers *et al.* (Proc. Natl. Acad. Sci. USA 82: 6512-6516 (1985)).

In addition, sequences have been characterized which cause the targeting of gene products to other cell compartments. Amino terminal sequences are responsible for targeting to the ER, the apoplast, and extracellular secretion from aleurone cells (Koehler & Ho, Plant Cell 2: 769-783 (1990)). Additionally, amino terminal sequences in conjunction with carboxy terminal sequences are responsible for vacuolar targeting of gene products (Shinshi *et al.* Plant Molec. Biol. 14: 357-368 (1990)).

By the fusion of the appropriate targeting sequences described above to transgene sequences of interest it is possible to direct the transgene product to any organelle or cell compartment. For chloroplast targeting, for example, the chloroplast signal sequence from the RUBISCO gene, the CAB gene, the EPSP synthase gene, or the GS2 gene is fused in frame to the amino terminal ATG of the transgene. The signal sequence selected should include the known cleavage site, and the fusion constructed should take into account any amino acids after the cleavage site which are required for cleavage. In some cases this requirement may be fulfilled by the addition of a small number of amino acids between the cleavage site and the transgene ATG or, alternatively, replacement of some amino acids within the transgene sequence. Fusions constructed for chloroplast import can be tested for efficacy of chloroplast uptake by *in vitro* translation of *in vitro* transcribed constructions followed by *in vitro* chloroplast uptake using techniques described by

Bartlett *et al.* In: Edelman *et al.* (Eds.) *Methods in Chloroplast Molecular Biology*, Elsevier pp 1081-1091 (1982) and Wasmann *et al.* *Mol. Gen. Genet.* 205: 446-453 (1986). These construction techniques are well known in the art and are equally applicable to mitochondria and peroxisomes.

The above-described mechanisms for cellular targeting can be utilized not only in conjunction with their cognate promoters, but also in conjunction with heterologous promoters so as to effect a specific cell-targeting goal under the transcriptional regulation of a promoter which has an expression pattern different to that of the promoter from which the targeting signal derives.

C. Transformation

Once the *NIM1* coding sequence has been cloned into an expression system, it is transformed into a plant cell. Plant tissues suitable for transformation include leaf tissues, root tissues, meristems, and protoplasts. The present system can be utilized in any plant which can be transformed and regenerated. Such methods for transformation and regeneration are well known in the art. Methodologies for the construction of plant expression cassettes as well as the introduction of foreign DNA into plants is generally described in the art. Generally, for the introduction of foreign DNA into plants, Ti plasmid vectors have been utilized for the delivery of foreign DNA. Also utilized for such delivery have been direct DNA uptake, liposomes, electroporation, microinjection, and microprojectiles. Such methods had been published in the art. See, for example, Bilang *et al.* (1991) *Gene* 100: 247-250; Scheid *et al.*, (1991) *Mol. Gen. Genet.* 228: 104-112; Guerche *et al.*, (1987) *Plant Science* 52: 111-116; Neuhauser *et al.*, (1987) *Theor. Appl. Genet.* 75: 30-36; Klein *et al.*, (1987) *Nature* 327: 70-73; Howell *et al.*, (1980) *Science* 208:1265; Horsch *et al.*, (1985) *Science* 227: 1229-1231; DeBlock *et al.*, (1989) *Plant Physiology* 91: 694-701; *Methods for Plant Molecular Biology* (Weissbach and Weissbach, eds.) Academic Press, Inc. (1988); and *Methods in Plant Molecular Biology* (Schuler and Zielinski, eds.) Academic Press, Inc. (1989). See also U.S. Patent Application No. 08/438,666, filed May 10, 1995, and U.S. Patent No. 5,625,136, both of which are incorporated herein by reference in their entirety. It is understood that the method of transformation will depend upon the plant cell to be transformed. Transformation of tobacco, tomato, potato, and *Arabidopsis thaliana* using a binary Ti vector system. *Plant Physiol.* 81:301-305, 1986; Fry, J., Barnason, A., and Horsch, R.B. Transformation of *Brassica napus* with *Agrobacterium tumefaciens* based vectors. *Pl. Cell Rep.* 6:321-325, 1987; Block, M.d. Genotype

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Bacteria from the genus *Agrobacterium* can be utilized to transform plant cells. Suitable species of such bacterium include *Agrobacterium tumefaciens* and *Agrobacterium rhizogens*. *Agrobacterium tumefaciens* (e.g., strains LBA4404 or EHA105) is particularly useful due to its
25 well-known ability to transform plants.

1. Transformation of Dicotyledons

Transformation techniques for dicotyledons are well known in the art and include *Agrobacterium*-based techniques and techniques which do not require *Agrobacterium*. Non-
30 *Agrobacterium* techniques involve the uptake of exogenous genetic material directly by protoplasts

or cells. This can be accomplished by PEG or electroporation mediated uptake, particle bombardment-mediated delivery, or microinjection. Examples of these techniques are described by Paszkowski *et al.*, EMBO J 3: 2717-2722 (1984), Potrykus *et al.*, Mol. Gen. Genet. 199: 169-177 (1985), Reich *et al.*, Biotechnology 4: 1001-1004 (1986), and Klein *et al.*, Nature 327: 70-73 (1987). In each case the transformed cells are regenerated to whole plants using standard techniques known in the art.

Agrobacterium-mediated transformation is a preferred technique for transformation of dicotyledons because of its high efficiency of transformation and its broad utility with many different species. The many crop species which are alfalfa and poplar (EP 0 317 511 (cotton [1313]), EP 0 249 432 (tomato, to Calgene), WO 87/07299 (*Brassica*, to Calgene), US 4,795,855 (poplar)). *Agrobacterium* transformation typically involves the transfer of the binary vector carrying the foreign DNA of interest (*e.g.* pCIB200 or pCIB2001) to an appropriate *Agrobacterium* strain which may depend of the complement of *vir* genes carried by the host *Agrobacterium* strain either on a co-resident Ti plasmid or chromosomally (*e.g.* strain CIB542 for pCIB200 and pCIB2001 (Uknes *et al.* Plant Cell 5: 159-169 (1993)). The transfer of the recombinant binary vector to *Agrobacterium* is accomplished by a triparental mating procedure using *E. coli* carrying the recombinant binary vector, a helper *E. coli* strain which carries a plasmid such as pRK2013 and which is able to mobilize the recombinant binary vector to the target *Agrobacterium* strain. Alternatively, the recombinant binary vector can be transferred to *Agrobacterium* by DNA transformation (Höfgen & Willmitzer, Nucl. Acids Res. 16: 9877 (1988)).

Transformation of the target plant species by recombinant *Agrobacterium* usually involves co-cultivation of the *Agrobacterium* with explants from the plant and follows protocols well known in the art. Transformed tissue is regenerated on selectable medium carrying the antibiotic or herbicide resistance marker present between the binary plasmid T-DNA borders.

Another approach to transforming plant cells with a gene involves propelling inert or biologically active particles at plant tissues and cells. This technique is disclosed in U.S. Patent Nos. 4,945,050; 5,036,006; and 5,100,792 all to Sanford et al. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and afford incorporation within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the

vector containing the desired gene. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried yeast cells, dried bacterium or a bacteriophage, each containing DNA sought to be introduced) can also be propelled into plant cell tissue.

5

2. Transformation of Monocotyledons

Transformation of most monocotyledon species has now also become routine. Preferred techniques include direct gene transfer into protoplasts using PEG or electroporation techniques, and particle bombardment into callus tissue. Transformations can be undertaken with a single DNA species or multiple DNA species (*i.e.* co-transformation) and both these techniques are suitable for use with this invention. Co-transformation may have the advantage of avoiding complete vector construction and of generating transgenic plants with unlinked loci for the gene of interest and the selectable marker, enabling the removal of the selectable marker in subsequent generations, should this be regarded desirable. However, a disadvantage of the use of co-transformation is the less than 100% frequency with which separate DNA species are integrated into the genome (Schocher *et al.* Biotechnology 4: 1093-1096 (1986)).

Patent Applications EP 0 292 435 (to Ciba-Geigy), EP 0 392 225 (to Ciba-Geigy) and WO 93/07278 (to Ciba-Geigy) describe techniques for the preparation of callus and protoplasts from an elite inbred line of maize, transformation of protoplasts using PEG or electroporation, and the regeneration of maize plants from transformed protoplasts. Gordon-Kamm *et al.* (Plant Cell 2: 603-618 (1990)) and Fromm *et al.* (Biotechnology 8: 833-839 (1990)) have published techniques for transformation of A188-derived maize line using particle bombardment. Furthermore, application WO 93/07278 (to Ciba-Geigy) and Koziel *et al.* (Biotechnology 11: 194-200 (1993)) describe techniques for the transformation of elite inbred lines of maize by particle bombardment. This technique utilizes immature maize embryos of 1.5-2.5 mm length excised from a maize ear 14-15 days after pollination and a PDS-1000He Biolistics device for bombardment.

Transformation of rice can also be undertaken by direct gene transfer techniques utilizing protoplasts or particle bombardment. Protoplast-mediated transformation has been described for Japonica-types and Indica-types (Zhang *et al.* Plant Cell Rep 7: 379-384 (1988); Shimamoto *et al.* Nature 338: 274-277 (1989); Datta *et al.* Biotechnology 8: 736-740 (1990)). Both types are also

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routinely transformable using particle bombardment (Christou *et al.* Biotechnology 9: 957-962 (1991)).

Patent Application EP 0 332 581 (to Ciba-Geigy) describes techniques for the generation, transformation and regeneration of Pooideae protoplasts. These techniques allow the transformation of *Dactylis* and wheat. Furthermore, wheat transformation has been described by Vasil *et al.* (Biotechnology 10: 667-674 (1992)) using particle bombardment into cells of type C long-term regenerable callus, and also by Vasil *et al.* (Biotechnology 11: 1553-1558 (1993)) and Weeks *et al.* (Plant Physiol. 102: 1077-1084 (1993)) using particle bombardment of immature embryos and immature embryo-derived callus. A preferred technique for wheat transformation, however, involves the transformation of wheat by particle bombardment of immature embryos and includes either a high sucrose or a high maltose step prior to gene delivery. Prior to bombardment, any number of embryos (0.75-1 mm in length) are plated onto MS medium with 3% sucrose (Murashiga & Skoog, Physiologia Plantarum 15: 473-497 (1962)) and 3 mg/l 2,4-D for induction of somatic embryos, which is allowed to proceed in the dark. On the chosen day of bombardment, embryos are removed from the induction medium and placed onto the osmoticum (*i.e.* induction medium with sucrose or maltose added at the desired concentration, typically 15%). The embryos are allowed to plasmolyze for 2-3 h and are then bombarded. Twenty embryos per target plate is typical, although not critical. An appropriate gene-carrying plasmid (such as pCIB3064 or pSG35) is precipitated onto micrometer size gold particles using standard procedures. Each plate of embryos is shot with the DuPont Biolistics® helium device using a burst pressure of ~1000 psi using a standard 80 mesh screen. After bombardment, the embryos are placed back into the dark to recover for about 24 h (still on osmoticum). After 24 hrs, the embryos are removed from the osmoticum and placed back onto induction medium where they stay for about a month before regeneration. Approximately one month later the embryo explants with developing embryogenic callus are transferred to regeneration medium (MS + 1 mg/liter NAA, 5 mg/liter GA), further containing the appropriate selection agent (10 mg/l basta in the case of pCIB3064 and 2 mg/l methotrexate in the case of pSOG35). After approximately one month, developed shoots are transferred to larger sterile containers known as "GA7s" which contain half-strength MS, 2% sucrose, and the same concentration of selection agent. Patent application 08/147,161 describes methods for wheat transformation and is hereby incorporated by reference.

Breeding

The isolated gene fragment of the present invention can be utilized to confer disease resistance to a wide variety of plant cells, including those of gymnosperms, monocots, and dicots.

5 Although the gene can be inserted into any plant cell falling within these broad classes, it is particularly useful in crop plant cells, such as rice, wheat, barley, rye, corn, potato, carrot, sweet potato, sugar beet, bean, pea, chicory, lettuce, cabbage, cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape,
10 raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tobacco, tomato, sorghum and sugarcane.

The high-level expression of the *NIM1* gene and mutants thereof necessary for constitutive expression of SAR genes, in combination with other characteristics important for production and quality, can be incorporated into plant lines through breeding. Breeding approaches and techniques
15 are known in the art. See, for example, Welsh J. R., Fundamentals of Plant Genetics and Breeding, John Wiley & Sons, NY (1981); Crop Breeding, Wood D. R. (Ed.) American Society of Agronomy Madison, Wisconsin (1983); Mayo O., The Theory of Plant Breeding, Second Edition, Clarendon Press, Oxford (1987); Singh, D.P., Breeding for Resistance to Diseases and Insect Pests, Springer-Verlag, NY (1986); and Wricke and Weber, Quantitative Genetics and Selection Plant Breeding,
20 Walter de Gruyter and Co., Berlin (1986).

Disease Resistance evaluation is performed by methods known in the art. For examples see, Uknes et al, (1993) *Molecular Plant Microbe Interactions* 6: 680-685; Gorlach et al., (1996) *Plant Cell* 8:629-643; Alexander et al., *Proc. Natl. Acad. Sci. USA* 90: 7327-7331.

EXAMPLES

The invention is illustrated in further detail by the following detailed procedures, preparations, and examples. The examples are for illustration only, and are not to be construed as limiting the scope of the present invention.

Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Sambrook, *et al.*, Molecular Cloning, eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989) and by T.J. Silhavy, M.L. Berman, and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and by Ausubel, F.M. *et al.*, Current Protocols in Molecular Biology, pub. by Greene Publishing Assoc. and Wiley-Interscience (1987).

A. Characterization of *nim1* Mutants

Example 1: Plant Lines and Fungal Strains

Arabidopsis thaliana ecotype Isilewskija (Ws-O; stock number CS 2360) and fourth-generation (T₄) seeds from T-DNA-transformed lines were obtained from the Ohio State University Arabidopsis Biological Resource Center (Columbus, OH). Second generation (M₂) seeds from ethyl methane sulfonate (EMS) mutagenized Ws-O plants were obtained from Lehle Seeds (Round Rock, TX).

Pseudomonas syringae pv. *Tomato* (*Pst*) strain DC3000 containing the cloned *avrRpt2* gene [DC3000(*avrRpt2*)] was obtained from B. Staskawicz, University of California, Berkeley. *P. parasitica* pathovars and their sources were as follows: Emwa from E. Holub and I.R. Crute, Horticultural Research Station, East Malling, Kent; Wela from A. Slusarenko and B. Mauch-Mani, Institut für Pflanzenbiologie, Zürich, Switzerland; and Noco from J. Parker, Sainsbury Laboratory, Norwich, England. Fungal cultures were maintained by weekly culturing on *Arabidopsis* ecotype Ws-O, Weiningen, and Col-O, for *P. parasitica* pathovars Emwa, Wela, and Noco, respectively.

Example 2: Mutant Screens

M₂ or T₄ seeds were grown on soil for 2 weeks under 14 hr of light per day, misted with 0.33 mM INA (0.25 mg/ml made from 25% INA in wettable powder; Ciba, Basel, Switzerland), and inoculated 4 days later by spraying a *P. parasitica* conidial suspension containing 5-10 × 10⁴ conidiospores per ml of water. This fungus is normally virulent on the Arabidopsis Ws-O ecotype, unless resistance is first induced in these plants with isonicotinic acid (INA) or a similar compound. Plants were kept under humid conditions at 18°C for 1 week and then scored for fungal sporulation. Plants that supported fungal growth after INA treatment were selected as putative mutants.

Following incubation in a high humidity environment, plants with visible disease symptoms were identified, typically 7 days after the infection. These plants did not show resistance to the fungus, despite the application of the resistance-inducing chemical and were thus potential *nim* (noninducible-immunity) mutant plants. From 360,000 plants, 75 potential *nim* mutants were identified.

These potential mutant plants were isolated from the flat, placed under low humidity conditions and allowed to set seed. Plants derived from this seed were screened in an identical manner for susceptibility to the fungus Emwa, again after pretreatment with INA. The progeny plants that showed infection symptoms were defined as *nim* mutants. Six *nim* lines were thus identified. One line (*nim1-1*) was isolated from the T-DNA population and five (*nim1-2*, *nim1-3*, *nim1-4*, *nim1-5*, and *nim1-6*) from the EMS population.

Example 3: Disease Resistance of *nim1* Plants

Salicylic acid (SA) and benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) are chemicals that, like INA, induce broad spectrum disease resistance (SAR) in wild type plants. Mutant plants were treated with SA, INA, and BTH and then assayed for resistance to *Peronospora parasitica*. *P. parasitica* isolate 'Emwa' is a *P.p.* isolate that is compatible in the Ws ecotype. Compatible isolates are those that are capable of causing disease on a particular host. The *P. parasitica* isolate 'Noco' is incompatible on Ws but compatible on the Columbia ecotype.

Incompatible pathogens are recognized by the potential host, eliciting a host response that prevents disease development.

Wild-type seeds and seeds for each of the *nim1* alleles (*nim1-1*, -2, -3, -4, -5, -6) were sown onto MetroMix 300 growing media, covered with a transparent plastic dome, and placed at 4°C in the dark for 3 days. After 3 days of 4°C treatment, the plants were moved to a phytotron for 2 weeks. By approximately 2 weeks post-planting, germinated seedlings had produced 4 true leaves. Plants were then treated with H₂O, 5mM SA, 300 µM BTH, or 300 µM INA. Chemicals were applied as a fine mist to completely cover the seedlings using a chromister. Water control plants were returned to the growing phytotron while the chemically treated plants were held in a separate but identical phytotron. At 3 days post-chemical application, water and chemically treated plants were inoculated with the compatible 'Emwa' isolate. 'Noco' inoculation was conducted on water treated plants only. Following inoculation, plants were covered with a clear plastic dome to maintain high humidity required for successful *P. parasitica* infection and placed in a growing chamber with 19°C day/17° C night temperatures and 8h light/16h dark cycles.

To determine the relative strength of the different *nim1* alleles, each mutant was microscopically analyzed at various timepoints after inoculation for the growth of *P. parasitica* under normal growth conditions and following pretreatment with either SA, INA, or BTH. Under magnification, sporulation of the fungus could be observed at very early stages of disease development. The percentage of plants/pot showing sporulation at 5d, 6d, 7d, 11d and 14d after inoculation was determined and the density of sporulation was also recorded.

Table 1 shows, for each of the *nim1* mutant plant lines, the percent of plants that showed some surface conidia on at least one leaf after infection with the Emwa race of *P. parasitica*. *P. parasitica* was inoculated onto the plants three days after water or chemical treatment. The table indicates the number of days after infection that the disease resistance was rated.

Table 1

Percent Infection - Emwa/Control

<u>mutant</u>	<u>Day 0</u>	<u>Day 5</u>	<u>Day 6</u>	<u>Day 7</u>	<u>Day 11</u>
Ws WT	0	10	25	100	90
<i>nim1-1</i>	0	75	95	100	100
<i>nim1-2</i>	0	30	85	100	100
<i>nim1-3</i>	0	30	90	100	100
<i>nim1-4</i>	0	80	100	100	100
<i>nim1-5</i>	0	0	5	100	100
<i>nim1-6</i>	0	5	70	80	100

Percent Infection - Emwa/SA

<u>mutant</u>	<u>Day 0</u>	<u>Day 5</u>	<u>Day 6</u>	<u>Day 7</u>	<u>Day 11</u>
Ws WT	0	5	30	70	100
<i>nim1-1</i>	0	5	95	100	100
<i>nim1-2</i>	0	5	95	100	100
<i>nim1-3</i>	0	10	90	100	100
<i>nim1-4</i>	0	75	100	100	100
<i>nim1-5</i>	0	0	20	75	100
<i>nim1-6</i>	0	80	100	100	100

Percent Infection - Emwa/INA

<u>mutant</u>	<u>Day 0</u>	<u>Day 5</u>	<u>Day 6</u>	<u>Day 7</u>	<u>Day 11</u>
Ws WT	0	0	0	0	0
<i>nim1-1</i>	0	5	80	100	100
<i>nim1-2</i>	0	15	95	100	100
<i>nim1-3</i>	0	10	60	100	100
<i>nim1-4</i>	0	80	100	100	100
<i>nim1-5</i>	0	0	0	5	5
<i>nim1-6</i>	0	1	50	90	100

Percent Infection - Emwa/BTH

<u>mutant</u>	<u>Day 0</u>	<u>Day 5</u>	<u>Day 6</u>	<u>Day 7</u>	<u>Day 11</u>
Ws WT	0	0	0	0	0
<i>nim1-1</i>	0	1	5	30	100
<i>nim1-2</i>	0	0	25	90	100
<i>nim1-3</i>	0	15	70	100	100
<i>nim1-4</i>	0	80	100	100	100
<i>nim1-5</i>	0	0	1	1	10
<i>nim1-6</i>	0	1	90	100	100

As shown in Table 1, during normal growth, *nim1-1*, *nim1-2*, *nim1-3*, *nim1-4*, and *nim1-6* all supported approximately the same rate of fungal growth, which was somewhat faster than the Ws-0 control. The exception was the *nim1-5* plants where fungal growth was delayed by several days relative to both the other *nim1* mutants and the Ws-0 control, but eventually all of the *nim1-5* plants succumbed to the fungus.

Following SA treatment, the mutants could be grouped into three classes: *nim1-4* and *nim1-6* showed a relatively rapid fungal growth; *nim1-1*, *nim1-2*, *nim1-3* plants exhibited a somewhat slower rate of fungal growth; and fungal growth in *nim1-5* plants was even slower than in the untreated Ws-0 controls. Following either INA or BTH treatment, the mutants also fell into three classes where *nim1-4* was the most severely compromised in its ability to restrict fungal growth following chemical treatment; *nim1-1*, *nim1-2*, *nim1-3*, and *nim1-6* were all moderately compromised; and *nim1-5* was only slightly compromised. In these experiments, Ws-0 did not support fungal growth following INA or BTH treatment. Thus, with respect to inhibition of fungal growth following chemical treatment, the mutants fell into three classes with *nim1-4* being the most severely compromised, *nim1-1*, *nim1-2*, *nim1-3* and *nim1-6* showing an intermediate inhibition of fungus and *nim1-5* with only slightly impaired fungal resistance.

Table 2 shows the disease resistance assessment via infection rating of the various *nim1* alleles as well as of NahG plants at 7 and 11 days after inoculation with *Peronospora parasitica*. WsWT indicates the Ws wild type parent line in which the *nim1* alleles were found. The various *nim1* alleles are indicated in the table and the NahG plant is indicated also.

A description of the NahG plant has been previously published. (Delaney et al., Science 266, pp. 1247-1250 (1994)). NahG *Arabidopsis* is also described in U.S. Patent No. 5,804,693, incorporated by reference herein. *nahG* is a gene from *Pseudomonas putida* encoding a salicylate hydroxylase that converts salicylic acid to catechol, thereby eliminating the accumulation of salicylic acid, a necessary signal transduction component for SAR in plants. Thus, NahG *Arabidopsis* plants do not display normal SAR, and they show much greater susceptibility in general to pathogens. However, the NahG plants still respond to the chemical inducers INA and BTH. NahG plants therefore serve as a kind of universal susceptibility control.

Table 2

Infection Severity - Emwa/Water

<u>mutant</u>	<u>Day 7</u>	<u>Day 11</u>
Ws WT	3	3
<i>nim1-1</i>	4	4.5
<i>nim1-2</i>	3	4
<i>nim1-3</i>	4	4
<i>nim1-4</i>	5	5
<i>nim1-5</i>	1	3.5
<i>nim1-6</i>	3	4.5
NahG	4	5

Infection Severity - Emwa/SA

<u>mutant</u>	<u>Day 7</u>	<u>Day 11</u>
Ws WT	3	4
<i>nim1-1</i>	3	4.5
<i>nim1-2</i>	3	4
<i>nim1-3</i>	3	4
<i>nim1-4</i>	4	5
<i>nim1-5</i>	3	3
<i>nim1-6</i>	4	4.5
NahG	4	5

Infection Severity - Emwa/INA

<u>mutant</u>	<u>Day 7</u>	<u>Day 11</u>
Ws WT	0	0
<i>nim1-1</i>	2.5	4
<i>nim1-2</i>	4	4
<i>nim1-3</i>	3	3.5
<i>nim1-4</i>	4	5
<i>nim1-5</i>	1	2
<i>nim1-6</i>	3	4.5
NahG	3	3

Infection Severity - Emwa/BTH

<u>mutant</u>	<u>Day 7</u>	<u>Day 11</u>
Ws WT	0	0
<i>nim1-1</i>	2.5	4
<i>nim1-2</i>	3.5	4
<i>nim1-3</i>	3	3.5
<i>nim1-4</i>	4	5
<i>nim1-5</i>	1.5	2
<i>nim1-6</i>	3	4
NahG	0	0

From Table 2 it can be seen that the *nim1-4* and *nim1-6* alleles had the most severe *Peronospora parasitica* infections; this was most easily observable at the earlier time points. In addition, the *nim1-5* allele showed the greatest response to both INA and BTH and therefore was deemed the weakest *nim1* allele. The NahG plants showed very good response to both INA and BTH and looked very similar to the *nim1-5* allele. However, at late time points, Day 11 in the Table, the disease resistance induced in the NahG plants began to fade, and there was a profound difference between INA and BTH in that the INA-induced resistance faded much faster and more severely than the resistance induced in the NahG plants by BTH. Also seen in these experiments was that INA and BTH induced very good resistance in Ws to Emwa, and the *nim1-1*, *nim1-2* and other *nim1* alleles showed virtually no response to SA or INA with regard to disease resistance.

The *nim1* plants' lack of responsiveness to the SAR-inducing chemicals SA, INA, and BTH implies that the mutation is downstream of the entry point(s) for these chemicals in the signal transduction cascade leading to systemic acquired resistance.

Example 4: Northern Analysis of SAR Gene Expression

Since SA, INA and BTH did not induce SAR, or SAR gene expression in any of the *nim1* plants, it was of interest to investigate whether pathogen infection could induce SAR gene expression in these plants, as it does in wild type plants. Thus, the accumulation of SAR gene mRNA was also used as a criterion to characterize the different *nim1* alleles.

Wild-type seeds and seeds for each of the *nim1* alleles (*nim1-1*, -2, -3, -4, -5, -6) were sown onto MetroMix 300 growing media, covered with a transparent plastic dome, and placed at 4°C in the dark for 3 days. After 3 days of 4°C treatment, the plants were moved to a phytotron for 2 weeks. By approximately two weeks post-planting, germinated seedlings had produced 4 true leaves. Plants were then treated with H₂O, 5mM SA, 300 µM BTH, or 300 µM INA. Chemicals were applied as a fine mist to completely cover the seedlings using a chromister. Water control plants were returned to the growing phytotron while the chemically treated plants were held in a separate but identical phytotron. At 3 days post-chemical application, water and chemically treated plants were inoculated with the compatible Emwa isolate. Noco inoculation was conducted on water treated plants only. Following inoculation, plants were covered with a clear plastic dome to

maintain high humidity required for successful *P. parasitica* infection and placed in a growing chamber with 19°C day/17°C night temperatures and 8h light/16h dark cycles. RNA was extracted from plants 3 days after either water or chemical treatment, or 14 days after inoculation with the compatible *P. parasitica* Emwa isolate. The RNA was size-fractionated by agarose gel electrophoresis and transferred to GeneScreenPlus membranes (DuPont).

Figures 1-3 present various RNA gel blots that indicate that SA, INA and BTH induce neither SAR nor SAR gene expression in *nim1* plants. In Figure 1, replicate blots were hybridized to *Arabidopsis* gene probes PR-1, PR-2 and PR-5 as described in Uknes *et al.* (1992). In contrast to the case in wild type plants, the chemicals did not induce RNA accumulation from any of these 3 SAR genes in *nim1-1* plants.

As shown in Figure 2, pathogen infection (Emwa) of wild type Ws-O plants induced PR-1 gene expression within 4 days after infection. In *nim1-1* plants, however, PR-1 gene expression was not induced until 6 days after infection and the level was reduced relative to the wild type at that time. Thus, following pathogen infection, PR-1 gene expression in *nim1-1* plants was delayed and reduced relative to the wild type.

The RNA gel blot in Figure 3 shows that *PR-1* mRNA accumulates to high levels following treatment of wild-type plants with SA, INA, or BTH or infection by *P. parasitica*. In the *nim1-1*, *nim1-2*, and *nim1-3* plants, *PR-1* mRNA accumulation was dramatically reduced relative to the wild type following chemical treatment. *PR-1* mRNA was also reduced following *P. parasitica* infection, but there was still some accumulation in these mutants. In the *nim1-4* and *nim1-6* plants, *PR-1* mRNA accumulation was more dramatically reduced than in the other alleles following chemical treatment (evident in longer exposures) and significantly less *PR-1* mRNA accumulated following *P. parasitica* infection, supporting the idea that these are particularly strong *nim1* alleles. *PR-1* mRNA accumulation was elevated in the *nim1-5* mutant, but only mildly induced following chemical treatment or *P. parasitica* infection. Based on both *PR-1* mRNA accumulation and fungal infection, the mutants have been determined to fall into three classes: severely compromised alleles (*nim1-4* and *nim1-6*); moderately compromised alleles (*nim1-1*, *nim1-2*, and *nim1-3*); and a weakly compromised allele (*nim1-5*).

Example 5: Determination of SA Accumulation in *nim1* Plants

Infection of wild type plants with pathogens that cause a necrotic reaction leads to accumulation of SA in the infected tissues. Endogenous SA is required for signal transduction in the SAR pathway, as breakdown of the endogenous SA leads to a decrease in disease resistance. This defines SA accumulation as a marker in the SAR pathway (Gaffney et al, 1993, Science 261, 754-756). The phenotype of *nim1* plants indicates a disruption in a component of the SAR pathway downstream of SA and upstream of SAR gene induction.

nim1 plants were tested for their ability to accumulate SA following pathogen infection. *Pseudomonas syringae* tomato strain DC 3000, carrying the *avrRpt2* gene, was injected into leaves of 4-week-old *nim1* plants. The leaves were harvested 2 days later for SA analysis as described by Delaney et al, 1995, PNAS 92, 6602-6606. This analysis showed that the *nim1* plants accumulated high levels of SA in infected leaves, as shown in Figure 4. Uninfected leaves also accumulated SA, but not to the same levels as the infected leaves, similar to what has been observed in wild-type *Arabidopsis*. This indicates that the *nim* mutation maps downstream of the SA marker in the signal transduction pathway. Furthermore, INA and BTH (inactive in *nim1* plants) have been demonstrated to stimulate a component in the SAR pathway downstream of SA (Vernooij et al. (1995); Friedrich, et al. (1996); and Lawton, et al. (1996)). In addition, as described above, exogenously applied SA did not protect *nim1* plants from Emwa infection.

Example 6: Genetic Analysis

To determine dominance of the various mutants that display the *nim1* phenotype, pollen from wild type plants was transferred to the stigmata of *nim1-1*, -2, -3, -4, -5, -6. If the mutation is dominant, then the *nim1* phenotype will be observed in the resulting F1 plants. If the mutation is recessive, then the resulting F1 plants will exhibit a wild type phenotype.

The data presented in Table 3 show that when *nim1-1*, -2, -3, -4 and -6 were crossed with the wild type, the resulting F1 plants exhibited the wild type phenotype. Thus, these mutations are recessive. In contrast, the *nim1-5* X wild type F1 progeny all exhibited the *nim1* phenotype, indicating that this is a dominant mutation. Following INA treatment, no *P. parasitica* sporulation

was observed on wild type plants, while the F1 plants supported growth and some sporulation of *P. parasitica*. However, the *nim1* phenotype in these F1 plants was less severe than observed when *nim1-5* was homozygous.

To determine allelism, pollen from the kanamycin-resistant *nim1-1* mutant plants was transferred to the stigmata of *nim1-2*, -3, -4, -5, -6. Seeds resulting from the cross were plated onto Murashige-Skoog B5 plates containing kanamycin at 25 µg/ml to verify the hybrid origin of the seed. Kanamycin resistant (F1) plants were transferred to soil and assayed for the *nim1* phenotype. Because the F1 progeny of the cross of the *nim1-5* mutant with the Ws wild type displays a *nim1* phenotype, analysis of *nim1-5* X *nim1-1* F2 was also carried out.

As shown in Table 3, all of the resulting F1 plants exhibited the *nim1* phenotype. Thus, the mutation in the *nim1-2*, -3, -4, -5, -6 was not complemented by the *nim1-1*; these plants all fall within the same complementation group and are therefore allelic. F2 progeny from the *nim1-5* X *nim1-1* cross also displayed the *nim1* phenotype, confirming that *nim1-5* is a *nim1* allele.

Table 3. Genetic Segregation of *nim* Mutants

Mutant	Generation	Female	Male	Phenotype	
				Wild type ^a	<i>nim1</i> ^b
<i>nim1-1</i>	F1	wild type ^c	<i>nim1-1</i>	24	0
	F2			98	32
<i>nim1-2</i>	F1	<i>nim1-2</i>	Wild type	3	0
<i>nim1-3</i>	F1	<i>nim1-3</i>	Wild type	3	0
<i>nim1-4</i>	F1	<i>nim1-4</i>	Wild type	3	0
<i>nim1-5</i>	F1	<i>nim1-5</i>	Wild type	0	35
	F1	Wild type	<i>nim1-5</i>	0	18
<i>nim1-6</i>	F1	<i>nim1-6</i>	Wild type	3	0
<i>nim1-2</i>	F1	<i>nim1-2</i>	<i>nim1-1</i>	0	15
<i>nim1-3</i>	F1	<i>nim1-3</i>	<i>nim1-1</i>	0	10
<i>nim1-4</i>	F1	<i>nim1-4</i>	<i>nim1-1</i>	0	15
<i>nim1-5</i>	F1	<i>nim1-5</i>	<i>nim1-1</i>	0	14
	F2			9	85
<i>nim1-6</i>	F1	<i>nim1-6</i>	<i>nim1-1</i>	0	12

^a Number of plants with elevated PR-1 mRNA accumulation and absence of *P. parasitica* after INA treatment.
^b Number of plants with no PR-1 mRNA accumulation and presence of *P. parasitica* after INA treatment.
^c Wild type denotes the wild type Ws-0 strain.

B. Mapping of the *nim1* Mutation

Mapping of the *nim1* mutation is described in exhaustive detail in Applicants' U.S. Patent Application Serial No. 08/773,559, filed December 27, 1996, which is incorporated by reference
5 herein in its entirety.

Example 7: Identification of Markers in and Genetic Mapping of the *NIM1* Locus

To determine a rough map position for *NIM1*, 74 *F₂* *nim* plants from a cross between *nim1-1* (Ws-0) and Landsberg *erecta* (*Ler*) were identified for their susceptibility to *P. parasitica* and lack of accumulation of *PR-1* mRNA following INA treatment. Using simple sequence length polymorphism (SSLP) markers (Bell and Ecker 1994), *nim1-1* was determined to lie about 8.2 centimorgans (cM) from *nga128* and 8.2 cM from *nga111* on the lower arm of chromosome 1. In addition, *nim1-1* was determined to lie between *nga111* and about 4 cM from the SSLP marker ATHGENEA. (Figure 5A)
15

For fine structure mapping, 1138 *nim* plants from an *F₂* population derived from a cross between *nim1-1* and *Ler* DP23 were identified based on both their inability to accumulate *PR-1* mRNA and their ability to support fungal growth following INA treatment. DNA was extracted from these plants and scored for zygosity at both ATHGENEA and *nga111*. As shown in Figure 5A, 93 recombinant chromosomes were identified between ATHGENEA and *nim1-1*, giving a genetic distance of approximately 4.1 cM (93 of 2276), and 239 recombinant chromosomes were identified between *nga111* and *nim1-1*, indicating a genetic distance of about 10.5 cM (239 of 2276). Informative recombinants in the ATHGENEA to *nga111* interval were further analyzed using amplified fragment length polymorphism (AFLP) analysis (Vos et al., 1995).
20

AFLP markers between ATHGENEA and *nga111* were identified and were used to construct a low resolution map of the region (Figures 5A and 5B). AFLP markers W84.2 (1 cM from *nim1-1*) and W85.1 (0.6 cM from *nim1-1*) were used to isolate yeast artificial chromosome (YAC) clones from the CIC (for Centre d'Etude du Polymorphisme Humain, INRA and CNRS) library (Creusot et al., 1995). Two YAC clones, CIC12H07 and CIC12F04, were identified with W84.2 and two YAC clones CIC7E03 and CIC10G07 were identified with the W85.1 marker. (Figure 5B) To bridge the gap between the two sets of flanking YAC clones, bacterial artificial
25
30

chromosome (BAC) and P1 clones that overlapped CIC12H07 and CIC12F04 were isolated and mapped, and sequential walking steps were carried out extending the BAC/P1 contig toward *NIM1* (Figure 5C; Liu et al., 1995; Chio et al., 1995). New AFLP's were developed during the walk that were specific for BAC or P1 clones, and these were used to determine whether the *NIM1* gene had been crossed. *NIM1* had been crossed when BAC and P1 clones were isolated that gave rise to both AFLP markers L84.6a and L84.8. The AFLP marker L84.6a found on P1 clones P1-18, P1-17, and P1-21 identified three recombinants and L84.8 found on P1 clones P1-20, P1-22, P1-23, and P1-24 and BAC clones, BAC-04, BAC-05, and BAC-06 identified one recombinant. Because these clones overlapped to form a large contig (>100 kb), and included AFLP markers that flanked *nim1*, the gene was determined to be located on the contig. The BAC and P1 clones that comprised the contig were used to generate additional AFLP markers, which showed that *nim1* was located between L84.Y1 and L84.8, representing a gap of about 0.09 cM.

C. Isolation of the *NIM1* Gene

Example 8: Construction of a Cosmid Contig

A cosmid library of the *NIM1* region was constructed in the *Agrobacterium*-compatible T-DNA cosmid vector pCLD04541 using CsCl-purified DNA from BAC-06, BAC-04, and P1-18. The DNAs of the three clones were mixed in equimolar quantities and were partially digested with the restriction enzyme *Sau3A*. The 20-25 kb fragments were isolated using a sucrose gradient, pooled and filled in with dATP and dGTP. Plasmid pCLD04541 was used as T-DNA cosmid vector. This plasmid contains a broad host range pRK290-based replicon, a tetracycline resistance gene for bacterial selection and the *nptII* gene for plant selection. The vector was cleaved with *XhoI* and filled in with dCTP and dTTP. The prepared fragments were then ligated into the vector. The ligation mix was packaged and transduced into *E. coli* strain XL1-blue MR (Stratagene). Resulting transformants were screened by hybridization with the BAC04, BAC06 and P1-18 clones and positive clones isolated. Cosmid DNA was isolated from these clones and template DNA was prepared using the ECs *EcoRI*/*MseI* and *HindIII*/*MseI*. The resulting AFLP fingerprint patterns were analyzed to determine the order of the cosmid clones. A set of 15 semi-overlapping cosmids

was selected spanning the *nim* region (Figure 5D). The cosmid DNAs were also restricted with EcoRI, PstI, BssHII and SgrAI. This allowed for the estimation of the cosmid insert sizes and the verification of the overlaps between the various cosmids as determined by AFLP fingerprinting.

Physical mapping showed that the physical distance between L84.Y1 and L84.8 was >90 kb, giving a genetic to physical distance of ~1 megabase per cM. To facilitate the identification of the *NIM1* gene, the DNA sequence of BAC04 was determined.

Example 9: Identification of a Clone containing the *NIM1* Gene.

Cosmids generated from clones spanning the *NIM1* region were moved into *Agrobacterium tumefaciens* AGL-1 through conjugative transfer in a tri-parental mating with helper strain HB101 (pRK2013). These cosmids were then used to transform a kanamycin-sensitive *nim1-1* Arabidopsis line using vacuum infiltration (Bechtold et al., 1993; Mindrinos et al., 1994). Seed from the infiltrated plants was harvested and allowed to germinate on GM agar plates containing 50 mg/ml kanamycin as a selection agent. Only plantlets that were transformed with cosmid DNA could detoxify the selection agent and survive. Seedlings that survived the selection were transferred to soil approximately two weeks after plating and tested for the *nim1* phenotype as described below. Transformed plants that no longer had the *nim1* phenotype identified cosmid(s) containing a functional *NIM1* gene.

Example 10: Complementation of the *nim1* Phenotype

Plants transferred to soil were grown in a phytotron for approximately one week after transfer. 300µm INA was applied as a fine mist to completely cover the plants using a chromister. After two days, leaves were harvested for RNA extraction and PR-1 expression analysis. The plants were then sprayed with *Peronospora parasitica* (isolate Emwa) and grown under high humidity conditions in a growing chamber with 19°C day/17°C night temperatures and 8h light/16h dark cycles. Eight to ten days following fungal infection, plants were evaluated and scored positive or negative for fungal growth. Ws and *nim1* plants were treated in the same way to serve as controls for each experiment.

Total RNA was extracted from the collected tissue using a LiCl/phenol extraction buffer (Verwoerd, et al. 1989). RNA samples were run on a formaldehyde agarose gel and blotted to GeneScreen Plus (DuPont) membranes. Blots were hybridized with a ³²P-labeled PR-1 cDNA probe. The resulting blots were exposed to film to determine which transformants were able to induce PR-1 expression after INA treatment. The results are summarized in Table 4, which shows complementation of the *nim1* phenotype by cosmid clones D5, E1, and D7.

Table 4

Clone Name	# of transformants	# of plants with INA induced PR-1/total # of plants tested (%)
A8	3	0/3 (0%)
A11	8	4/18 (22%)
C2	10	1/10 (10%)
C7	33	1/32 (3%)
D2	81	4/49 (8%)
D5	6	5/6 (83%)
E1	10	10/10 (100%)
D7	129	36/36 (100%)
E8	9	0/9 (0%)
F12	6	0/6 (0%)
E6	1	0/1 (0%)
E7	34	0/4 (0%)
WS-control (wild-type)	NA	28/28 (100%)
<i>nim1-1</i> phenotype control	NA	0/34 (0%)

NA - not applicable

Example 11: Sequencing of the *NIM1* Gene Region

BAC04 DNA (25 ug, obtained from KeyGene) was the source of DNA used for sequence analysis, as this BAC was the clone completely encompassing the region that complemented the *nim1* mutants. BAC04 DNA was randomly sheared in a nebulizer to generate fragments with an average length of about 2 kb. Ends of the sheared fragments were repaired, and the fragments were purified. Prepared DNA was ligated with EcoRV-digested pBRKanF4 (a derivative of pBRKan_{F1} (Bhat 1993)). Resulting kanamycin-resistant colonies were selected for plasmid isolation using the Wizard Plus 9600 Miniprep System (Promega). Plasmids were sequenced using dye terminator

chemistry (Applied BioSystems, Foster City, CA) with primers designed to sequence both strands of the plasmids (M13-21 forward and T7 reverse, Applied BioSystems). Data was collected on ABI377 DNA sequencers. Sequences were edited and assembled into contigs using Sequencher 3.0 (GeneCodes Corp., Ann Arbor, MI), the Staden genome assembly programs, phred, phrap and crossmatch (Phil Green, Washington University, St. Louis, MO) and consed (David Gordon, Washington University, St. Louis, MO). DNA from the cosmids found to complement the *nim1-1* mutation was sequenced using primers designed by Oligo 5.0 Primer Analysis Software (National Biosciences, Inc., Plymouth, MN). Sequencing of DNA from Ws-0 and the *nim1* alleles and cDNAs was performed essentially as described above.

A region of approximately 9.9 kb defined by the overlap of cosmids E1 and D7 was identified by complementation analysis to contain the *nim1* region. Primers that flanked the insertion site of the vector and that were specific to the cosmid backbone were designed using Oligo 5.0 Primer Analysis Software (National Biosciences, Inc.). DNA was isolated from cosmids D7 and E1 using a modification of the ammonium acetate method (Traynor, P.L., 1990. BioTechniques 9(6): 676.) This DNA was directly sequenced using Dye Terminator chemistry above. The sequence obtained allowed determination of the endpoints of the complementing region. The region defined by the overlap of cosmids E1 and D7 is presented as SEQ ID NO:1.

A truncated version of the BamHI-EcoRV fragment was also constructed, resulting in a construct that contained none of the "Gene 3" region (Fig. 5D). The following approach was necessary due the presence of HindIII sites in the Bam-Spe region of the DNA. The BamHI-EcoRV construct was completely digested with SpeI, then was split into two separate reactions for double digestion. One aliquot was digested with BamHI, the other HindIII. A BamHI-SpeI fragment of 2816 bp and a HindIII-SpeI fragment of 1588 bp were isolated from agarose gels (QiaQuick Gel extraction kit) and were ligated to BamHI-HindIII-digested pSGCG01. DH5a was transformed with the ligation mix. Resulting colonies were screened for the correct insert by digestion with HindIII following preparation of DNA using Wizard Magic MiniPreps (Promega). A clone containing the correct construct was electroporated into *Agrobacterium* strain GV3101 for transformation of *Arabidopsis* plants.

Example 12: Sequence Analysis and Subcloning of the *NIM1* Region

The 9.9 kb region containing the *NIM1* gene was analyzed for the presence of open reading frames in all six frames using Sequencher 3.0 and the GCG package. Four regions containing large ORF's were identified as possible genes (Gene Regions 1-4 in Figure 5D). These four regions were PCR amplified from DNA of the wild-type parent and the six different *nim1* allelic variants *nim1-1*, -2, -3, -4, -5, and -6. Primers for these amplifications were selected using Oligo 5.0 (National Biosciences, Inc.) and were synthesized by Integrated DNA Technologies, Inc. PCR products were separated on 1.0% agarose gels and were purified using the QIAquick Gel Extraction Kit. The purified genomic PCR products were directly sequenced using the primers used for the initial amplification and with additional primers designed to sequence across any regions not covered by the initial primers. Average coverage for these gene regions was approximately 3.5 reads/base.

Sequences were edited and were assembled using Sequencher 3.0. Base changes specific to various *nim1* alleles were identified only in the region designated Gene Region 2, as shown below in Table 5, which shows sequence variations among all six of the *nim1* alleles.

Table 5

Allele/ ecotype	Gene Region			
	1 (bases 590- 1090)	2 (<i>NIM1</i>) (bases 1380-4100)	3 (bases 5870 - 6840)	4 (bases 8140- 9210)
<i>nim1-1</i>	no changes	t inserted at 2981: change of 7AA and premature termination of protein.	no changes	no changes
<i>nim1-2</i>	no changes	g to a at 2799: His to Tyr	no changes	no changes
<i>nim1-3</i>	no changes	deletion of t at 3261: change of 10AA and premature termination of protein.	no changes	no changes
<i>nim1-4</i>	no changes	c to t at 2402: Arg to lys	no changes	no changes
<i>nim1-5</i>	no changes	c to t at 2402: Arg to lys	no changes	no changes
<i>nim1-6</i>	g to a at 734: asp to lys	g to a at 2670: Gln to Stop	no changes	no changes
WS (compared to Columbia)	no changes	a to g at 1607: Ile to Leu a to c at 2344: intron t to g at 2480: Gln to Pro g to c at 2894: Ser to Trp ggc deleted at 3449: lose Ala c to t at 3490: Ala to Thr c to t at 3498: Ser to Asn a to t at 3873: non-coding g to a at 3992: non-coding g to a at 4026: non-coding g to a at 4061: non-coding	t to a at 5746 a to t at 5751 t to a at 5754 c to t at 6728 a to t at 6815 t to c at 6816	t to g at 8705 g to t at 8729 g to t at 8739 g to t at 8784 c to a at 8789 c to t at 8812 a to g at 8829 t to g at 8856 a to c at 9004 a to t at 9011 a to g at 8461
RNA detected	No	Yes	No	No

Positions listed in the table relate to SEQ ID NO:1. All alleles *nim1-1* to *nim1-6* are WS strain. Columbia-0 represents the wild type

It is apparent that the *NIM1* gene lies within Gene Region 2, because there are amino acid changes or alterations of sequence within the open reading frame of Gene Region 2 in all six *nim1* alleles. At the same time, at least one of the *nim1* alleles shows no changes in the open reading frames within Gene Regions 1, 3 and 4. Therefore, the only gene region within the 9.9 kb region that could contain the *NIM1* gene is Gene Region 2.

The Ws section of Table 5 indicates the changes in the Ws ecotype of *Arabidopsis* relative to the Columbia ecotype of *Arabidopsis*. The sequences presented herein relate to the Columbia

ecotype of *Arabidopsis*, which contains the wild type gene in the experiments described herein. The changes are listed as amino acid changes within Gene Region 2 (the *NIM1* region) and are listed as changes in base pairs in the other regions.

The cosmid region containing the *nim1* gene was delineated by a BamH1-EcoRV restriction fragment of ~5.3 kb. Cosmid DNA from D7 and plasmid DNA from pBlueScriptII(pBSII) were digested with BamHI and with EcoRV (NEB). The 5.3 kb fragment from D7 was isolated from agarose gels and was purified using the QIAquick gel extraction kit (# 28796, Qiagen). The fragment was ligated overnight to the Bam-EcoRV-digested pBSII and the ligation mixture was transformed into *E. coli* strain DH5a. Colonies containing the insert were selected, DNA was isolated, and confirmation was made by digestion with HindIII. The Bam-EcoRV fragment was then engineered into a binary vector (pSGCG01) for transformation into *Arabidopsis*.

Example 13: Northern Analysis of the Four Gene Regions

Identical Northern blots were made from RNA samples isolated from water-, SA-, BTH- and INA-treated Ws and *nim1* lines as previously described in Delaney, et al. (1995). These blots were hybridized with PCR products generated from the four gene regions identified in the 9.9 kb *NIM1* gene region (SEQ ID NO:1). Only the gene region containing the *NIM1* gene (Gene Region 2) had detectable hybridization with the RNA samples, indicating that only the *NIM1* region contains a detectable transcribed gene (Figure 5D and Table 5).

Example 14: Complementation with Gene Region 2

Gene Region 2 (Fig. 5D) was also demonstrated to contain the functional *NIM1* gene by doing additional complementation experiments. A BamHI/HindIII genomic DNA fragment containing Gene Region 2 was isolated from cosmid D7 and was cloned into the binary vector pSGCG01 containing the gene for kanamycin resistance. The resulting plasmid was transformed into the *Agrobacterium* strain GV3101 and positive colonies were selected on kanamycin. PCR was used to verify that the selected colony contains the plasmid. Kanamycin-sensitive *nim1-1* plants were infiltrated with this bacteria as previously described. The resulting seed was harvested

and planted on GM agar containing 50µg/ml kanamycin. Plants surviving selection were transferred to soil and tested for complementation. Transformed plants and control Ws and *nim1* plants were sprayed with 300µm INA. Two days later, leaves were harvested for RNA extraction and PR-1 expression analysis. The plants were then sprayed with *Peronospora parasitica* (isolate Emwa) and grown as previously described. Ten days following fungal infection, plants were evaluated and scored positive or negative for fungal growth. All of the 15 transformed plants, as well as the Ws controls, were negative for fungal growth following INA treatment, while the *nim1* controls were positive for fungal growth. RNA was extracted and analyzed as described above for these transformants and controls. Ws controls and all 15 transformants showed PR-1 gene induction following INA treatment, while the *nim1* controls did not show PR-1 induction by INA.

Example 15: Isolation of a *NIM1* cDNA

An Arabidopsis cDNA library made in the IYES expression vector (Elledge et al, 1991, PNAS 88, 1731-1735) was plated and plaque lifts were performed. Filters were hybridized with a ³²P-labeled PCR product generated from Gene Region 2 (Figure 5D). 14 positives were identified from a screen of approximately 150,000 plaques. Each plaque was purified and plasmid DNA was recovered. cDNA inserts were digested out of the vector using EcoRI, agarose-gel-purified and sequenced. Sequence obtained from the longest cDNA is indicated in SEQ ID NO:2 and Figure 6. To confirm that the 5' end of the cDNA had been obtained, a Gibco BRL 5' RACE kit was used following manufacturer's instructions. The resulting RACE products were sequenced and found to include the additional bases indicated in Figure 6. The transcribed region present in both cDNA clones and detected in RACE is shown as capital letters in Figure 6. Changes in the alleles are shown above the DNA strand. Capitals indicate the presence of the sequence in a cDNA clone or detected after RACE PCR.

The same RNA samples produced in the induction studies (Figure 3) were also probed with the *NIM1* gene using a full-length cDNA clone as a probe. In Figure 7 it can be seen that INA induced the *NIM1* gene in the wild type Ws allele. However, the *nim1-1* mutation allele showed a lower basal level expression of the *NIM1* gene, and it was not inducible by INA. This was similar to what was observed in the *nim1-3* allele and the *nim1-6* allele. The *nim1-2* allele showed

approximately normal levels in the untreated sample and showed similar induction to that of the wild type sample, as did the *nim1-4* allele. The *nim1-5* allele seemed to show higher basal level expression of the *NIM1* gene and much stronger expression when induced by chemical inducers.

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D. *NIM1* Homologues

Example 16: BLAST Search with the *NIM1* Sequence

A multiple sequence alignment was constructed using Clustal V (Higgins, Desmond G. and Paul M. Sharp (1989), Fast and sensitive multiple sequence alignments on a microcomputer, CABIOS 5:151-153) as part of the DNA* (1228 South Park Street, Madison Wisconsin, 53715) Lasergene Biocomputing Software package for the Macintosh (1994). Certain regions of the *NIM1* protein are homologous in amino acid sequence to 4 different rice cDNA protein products. The homologies were identified using the *NIM1* sequences in a GenBank BLAST search. Comparisons of the regions of homology in *NIM1* and the rice cDNA products are shown in Figure 8 (*See also*, SEQ ID NO:3 and SEQ ID NO's: 4-11). The *NIM1* protein fragments show from 36 to 48% identical amino acid sequences with the 4 rice products.

Example 17: Isolation of Homologous Genes from Other Plants

Using the *NIM1* cDNA as a probe, homologs of Arabidopsis *NIM1* are identified through screening genomic or cDNA libraries from different crops such as, but not limited to those listed below in Example 22. Standard techniques for accomplishing this include hybridization screening of plated DNA libraries (either plaques or colonies; see, *e.g.* Sambrook *et al.*, Molecular Cloning, eds., Cold Spring Harbor Laboratory Press. (1989)) and amplification by PCR using oligonucleotide primers (see, *e.g.* Innis *et al.*, PCR Protocols, a Guide to Methods and Applications eds., Academic Press (1990)). Homologs identified are genetically engineered into the expression vectors herein and transformed into the above listed crops. Transformants are evaluated for enhanced disease resistance using relevant pathogens of the crop plant being tested.

NIM1 homologs in the genomes of cucumber, tomato, tobacco, maize, wheat and barley have been detected by DNA blot analysis. Genomic DNA was isolated from cucumber, tomato, tobacco, maize, wheat and barley, restriction digested with the enzymes BamHI, HindIII, XbaI, or SalI, electrophoretically separated on 0.8% agarose gels and transferred to nylon membrane by capillary blotting. Following UV-crosslinking to affix the DNA, the membrane was hybridized under low stringency conditions [(1%BSA; 520mM NaPO₄, pH7.2; 7% lauryl sulfate, sodium salt; 1mM EDTA; 250 mM sodium chloride) at 55°C for 18-24h] with ³²P-radiolabelled *Arabidopsis thaliana* *NIM1* cDNA. Following hybridization the blots were washed under low stringency conditions [6XSSC for 15 min. (X3) 3XSSC for 15 min. (X1) at 55°C; 1XSSC is 0.15M NaCl, 15mM Na-citrate (pH7.0)] and exposed to X-ray film to visualize bands that correspond to *NIM1*.

In addition, expressed sequence tags (EST) identified with similarity to the *NIM1* gene such as the rice EST's described in Example 16 can also be used to isolate homologues. The rice EST's may be especially useful for isolation of *NIM1* homologues from other monocots.

Homologues may be obtained by PCR. In this method, comparisons are made between known homologues (e.g., rice and *Arabidopsis*). Regions of high amino acid and DNA similarity or identity are then used to make PCR primers. Regions rich in M and W are best followed by regions rich in F, Y, C, H, Q, K and E because these amino acids are encoded by a limited number of codons. Once a suitable region is identified, primers for that region are made with a diversity of substitutions in the 3rd codon position. This diversity of substitution in the third position may be constrained depending on the species that is being targeted. For example, because maize is GC rich, primers are designed that utilize a G or a C in the 3rd position, if possible.

The PCR reaction is performed from cDNA or genomic DNA under a variety of standard conditions. When a band is apparent, it is cloned and/or sequenced to determine if it is a *NIM1* homologue.

E. High-Level Expression of *NIM1* Confers Disease Resistance In Plants

High-level expression of the *NIM1* gene in transgenic plants to confer a CIM phenotype is also described in Applicants' U.S. Patent Application Serial No. 08/773,554, filed December 27, 1996, which is incorporated by reference herein in its entirety.

Example 18: High-Level Expression of *NIM1* Due To Insertion Site Effect

To determine if any of the transformants described above in Example 10/Table 4 had high-level expression of *NIM1* due to insertion site effect, primary transformants containing the D7, D5 or E1 cosmids (containing the *NIM1* gene) were selfed and the T2 seed collected. Seeds from one E1 line, four D5 lines and 95 D7 lines were sown on soil and grown as described above. When the T2 plants had obtained at least four true leaves, a single leaf was harvested separately for each plant. RNA was extracted from this tissue and analyzed for PR-1 and *NIM1* expression. Plants were then inoculated with *P. parasitica* (Emwa) and analyzed for fungal growth at 3-14 days, preferably 7-12 days, following infection. Plants showing higher than normal *NIM1* and PR-1 expression and displaying fungal resistance demonstrated that high-level expression of *NIM1* confers a CIM phenotype.

Table 6 shows the results of testing of various transformants for resistance to fungal infection. As can be seen from the table, a number of transformants showed less than normal fungal growth and several showed no visible fungal growth at all. RNA was prepared from collected samples and analyzed as previously described (Delaney et al, 1995). Blots were hybridized to the *Arabidopsis* gene probe PR-1 (Uknes et al, 1992). Lines D7-74, D5-6 and E1-1 showed early induction of PR-1 gene expression, whereby PR-1 mRNA was evident by 24 or 48 hours following fungal treatment. These three lines also demonstrated resistance to fungal infection.

Table 6

Line	P.parasitica growth	Line	P.parasitica growth	Line	P.parasitica growth
D7-2	negative	52	+	90	+
3	+	53	+	91	+
9	+	54	+/-	92	+
11	+	56	+	93	+
12	+	57	+	94	+
13	+	58	+	95	+
14	+	59	+	96	+
17	+	60	+	97	+
18	+	61	+	98	+/-
19	+	62	+	100	+/-
20	+	63	+	101	+/-
21	+	64	+	102	+/-
22	+	66	+	103	+
23	+	67	+	104	+
24	+	68	+	106	+
25	+	69	+	107	+
28	+	70	+	108	+
29	+	71	+	114	+
31	+	72	+	115	+
32	+	73	+	118	+
33	+	74	negative	119	+
34	+	75	+	122	+
35	+	77	+	123	+
36	+	78	+	124	+
38	+	79	+	125	+
39	+	80	+/-	126	+
42	+	81	+	128	+
43	+	82	+	129	+
46	+	83	+	130	+
47	+	84	+	D5-1	+
48	+	85	+	2	+
49	+	86	+	4	+
50	+	87	+/-	6	+/-
51	+	89	negative	E1-1	negative

Plants were treated with P. parasitica isolate Emwa and scored 10 days later.

+, normal fungal growth

5 +/-, less than normal fungal growth

negative, no visible fungal growth

Example 19: 35S Driven Expression of *NIM1*

Plants constitutively expressing the *NIM1* gene are also generated from transformation of Ws or Col wild type plants with the BamHI-HindIII *Arabidopsis* genomic fragment (Figure 6) cloned into pSGCG01 transformed into the Agrobacterium strain GV3101 (pMP90, Koncz and Schell, 1986, Mol. Gen. Genet. 204:383-396). In another construct, the full-length *NIM1* cDNA is cloned into the EcoRI site of pCGN1761 ENX (Comai et al, 1990, Plant Mol. Biol. 15, 373-381). From the resulting plasmid, an XbaI fragment containing an enhanced CaMV 35S promoter, the *NIM1* cDNA in the correct orientation for transcription and a *tml* 3' terminator is obtained. This fragment is cloned into the binary vector pCIB200 and transformed into GV3101. Ws or Col plants are infiltrated as previously described with each of these transformed strains. The resulting seed is harvested and plated on GM agar containing 50 mg/ml kanamycin. Surviving plantlets are transferred to soil and tested for a disease resistant phenotype.

Example 20: Generation of Altered *NIM1* Forms Using the *NIM1* Gene and 35S Promoter

A BamHI/HindIII *Arabidopsis* genomic fragment (Figure 6: bases 1249-5655) containing the *NIM1* promoter, gene and downstream sequence cloned into pSGCG01 is restriction endonuclease digested with PstI and the ends made blunt with T4 polymerase. The fragment is further restriction endonuclease digested with *SpeI* yielding a 2162 bp fragment. The *NIM1* cDNA cloned into the EcoRI site of pCGN1761ENX (see above) is restriction endonuclease digested with NotI and the ends made blunt with T4 polymerase. The fragment is further restriction endonuclease digested with *SpeI*. The 2162 bp genomic *NIM1* fragment from above is ligated with the pCGN1761ENX vector containing 91 bp of the *NIM1* cDNA. The transformation cassette including the 35S promoter and *tml* terminator is released from pCGN1761ENX by partial restriction digestion with XbaI and ligated into the XbaI site of dephosphorylated pCIB200. The resulting plasmid is transformed into GV3101 and Ws or Col plants are infiltrated as previously described. Seeds are plated, and surviving plantlets are selected as described above.

Example 21: Assessment of CIM Phenotype in the High-Level Expression Transformants of *NIM1*

A leaf from each primary transformant is harvested, RNA is isolated (Verwoerd et al., 1989, Nuc Acid Res, 2362) and tested for constitutive PR-1 expression and *NIM1* expression by RNA blot analysis (Uknes et al., 1992). Each transformant is evaluated for an enhanced disease resistance response indicative of constitutive SAR expression analysis (Uknes et al., 1992). Conidial suspensions of $5\text{-}10 \times 10^4$ spores/ml from two compatible *P. parasitica* isolates, Emwa and Noco (i.e. these fungal strains cause disease on wildtype Ws-O and Col-0 plants, respectively), are prepared and transformants are sprayed with the appropriate isolate depending on the ecotype of the transformant. Inoculated plants are incubated under high humidity for 7 days. Plants are disease rated at day 7 and a single leaf is harvested for RNA blot analysis utilizing a probe which provides a means to measure fungal infection.

Transformants that exhibit a CIM phenotype are taken to the F1 generation and homozygous plants are identified. Transformants are subjected to a battery of disease resistance tests. Fungal infection with Noco and Emwa are performed and leaves stained with lactophenol blue to identify the presence of fungal hyphae as described in Dietrich et al., (1994). Transformants are infected with the bacterial pathogen *Pseudomonas syringae* DC3000 to evaluate the spectrum of resistance evident as described in Uknes et al., (1993). Uninfected plants are evaluated for both free and glucose-conjugated SA and leaves are stained with lactophenol blue to evaluate for the presence of microscopic lesions. Resistant plants are sexually crossed with SAR mutants such as NahG and *ndr1* to establish the epistatic relationship of the resistance phenotype to other mutants and evaluate how these dominant negative mutants of *NIM1* may influence the SA-dependent feedback loop.

Example 22: High Level Expression of *NIM1* in Crop Plants

Those constructs conferring a CIM phenotype in Col-0 or Ws-0 and others are transformed into crop plants for evaluation. Although the *NIM1* gene can be inserted into any plant cell falling within these broad classes, it is particularly useful in crop plant cells, such as rice, wheat, barley, rye, corn, potato, carrot, sweet potato, sugar beet, bean, pea, chicory, lettuce, cabbage, cauliflower,

broccoli, turnip, radish, spinach, asparagus, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tobacco, tomato, sorghum and sugarcane. Transformants are evaluated for enhanced disease resistance. In a preferred embodiment of the invention, the expression of the *NIMI* gene is at a level which is at least two-fold above the expression level of the *NIMI* gene in wild type plants and is preferably ten-fold above the wild type expression level.

F. Other Uses of *nim* Phenotype Plants Generally

Example 23: The Use of *nim* Mutants in Disease Testing

nim mutants are challenged with numerous pathogens and found to develop larger lesions more quickly than wild-type plants. This phenotype is referred to as UDS (i.e. universal disease susceptibility) and is a result of the mutants failing to express SAR genes to effect the plant defense against pathogens. The UDS phenotype of *nim* mutants renders them useful as control plants for the evaluation of disease symptoms in experimental lines in field pathogenesis tests where the natural resistance phenotype of so-called wild type lines may vary (i.e. to different pathogens and different pathotypes of the same pathogen). Thus, in a field environment where natural infection by pathogens is being relied upon to assess the resistance of experimental lines, the incorporation into the experiment of *nim* mutant lines of the appropriate crop plant species would enable an assessment of the true level and spectrum of pathogen pressure, without the variation inherent in the use of non-experimental lines.

Example 24: Assessment of the Utility of Transgenes for the Purposes of Disease Resistance

nim mutants are used as host plants for the transformation of transgenes to facilitate their assessment for use in disease resistance. For example, an *Arabidopsis nim* mutant line, characterized by its UDS phenotype, is used for subsequent transformations with candidate genes for disease resistance thus enabling an assessment of the contribution of an individual gene to resistance against the basal level of the UDS *nim* mutant plants.

Example 25: *nim* Mutants as a Tool in Understanding Plant-Pathogen Interactions

nim mutants are useful for the understanding of plant pathogen interactions, and in particular for the understanding of the processes utilized by the pathogen for the invasion of plant cells. This is so because *nim* mutants do not mount a systemic response to pathogen attack, and the unabated development of the pathogen is an ideal scenario in which to study its biological interaction with the host.

Of further significance is the observation that a host *nim* mutant may be susceptible to pathogens not normally associated with that particular host, but instead associated with a different host. For example, an *Arabidopsis* *nim* mutant such as *nim1-1*, *-2*, *-3*, *-4*, *-5*, or *-6* is challenged with a number of pathogens that normally only infect tobacco, and found to be susceptible. Thus, the *nim* mutation causing the UDS phenotype leads to a modification of pathogen-range susceptibility and this has significant utility in the molecular, genetic and biochemical analysis of host-pathogen interaction.

Example 26: *nim* Mutants for Use in Fungicide Screening

nim mutants are particularly useful in the screening of new chemical compounds for fungicide activity. *nim* mutants selected in a particular host have considerable utility for the screening of fungicides using that host and pathogens of the host. The advantage lies in the UDS phenotype of the mutant that circumvents the problems encountered by the host being differentially susceptible to different pathogens and pathotypes, or even resistant to some pathogens or pathotypes. By way of example, *nim* mutants in wheat could be effectively used to screen for fungicides to a wide range of wheat pathogens and pathotypes as the mutants would not mount a resistance response to the introduced pathogen and would not display differential resistance to different pathotypes that might otherwise require the use of multiple wheat lines, each adequately susceptible to a particular test pathogen. Wheat pathogens of particular interest include (but are not limited to) *Erysiphe graminis* (the causative agent of powdery mildew), *Rhizoctonia solani* (the causative agent of sharp eyespot), *Pseudocercospora herpotrichoides* (the causative agent of

eyespot), *Puccinia spp.* (the causative agents of rusts), and *Septoria nodorum*. Similarly, *nim* mutants of corn would be highly susceptible to corn pathogens and therefore useful in the screening for fungicides with activity against corn diseases.

nim mutants have further utility for the screening of a wide range of pathogens and pathotypes in a heterologous host i.e. in a host that may not normally be within the host species range of a particular pathogen and that may be particularly easily to manipulate (such as *Arabidopsis*). By virtue of its UDS phenotype the heterologous host is susceptible to pathogens of other plant species, including economically important crop plant species. Thus, by way of example, the same *Arabidopsis nim* mutant could be infected with a wheat pathogen such as *Erysiphe graminis* (the causative agent of powdery mildew) or a corn pathogen such as *Helminthosporium maydis* and used to test the efficacy of fungicide candidates. Such an approach has considerable improvements in efficiency over currently used procedures of screening individual crop plant species and different cultivars of species with different pathogens and pathotypes that may be differentially virulent on the different crop plant cultivars. Furthermore, the use of *Arabidopsis* has advantages because of its small size and the possibility of thereby undertaking more tests with limited resources of space.

DEPOSITS

The following vector molecules have been deposited under the terms of the Budapest Treaty with American Type Culture Collection 12301 Parklawn Drive Rockville, MD 20852, U.S.A. on the dates indicated below:

Plasmid BAC-04 was deposited with ATCC on May 8, 1996 as ATCC 97543.

Plasmid P1-18 was deposited with ATCC on June 13, 1996 as ATCC 97606.

Cosmid D7 was deposited with ATCC on September 25, 1996 as ATCC 97736.

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The disclosures of each of the following references are hereby expressly incorporated by reference into the instant disclosure:

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